PROGENITOR CELL HETEROGENEITY IN THE DEVELOPING RETINA IS REVEALED THROUGH DISTINCT REQUIREMENTS FOR LHX2 IN THE REGULATION OF COMPETENCE AND PROLIFERATION

by

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ABSTRACT

During development, all seven of the major retinal cell types are produced in a distinct yet overlapping order from a single pool of multipotent retinal progenitor cells (RPCs). In order to accomplish this task, it is thought that each individual RPC proceeds irreversibly through a series of intrinsically defined competence states, capable of producing only a subset of these cell types at any given time. To ensure that all early- and late-born cell types are produced in the correct number, RPCs must not only proceed through these competence states in a timely fashion, but also limit their rate of differentiation in order to prevent premature depletion. *Lhx2* is a LIM-homeobox transcription factor expressed in many different tissues during development, known to regulate both proliferation and fate choice. It is also expressed in most if not all RPCs, and we assessed its contribution to their various properties by performing conditional inactivation at multiple time points during retinal neurogenesis. We find that *Lhx2* is required within a limited temporal window to ensure the balanced production of early-born cell types, as retinal ganglion cells (RGCs) are selectively overproduced in the *Lhx2* conditional knock-out (CKO) retina. LHX2 is also necessary for the normal cessation of RGC genesis, suggesting that in its absence, RPCs fail to undergo a normal transition in competence. We show that sustained Notch signaling, dependent on the transcription factor *Rbpj*, is likely responsible for maintaining a low level of both proliferation and neurogenesis in the absence of *Lhx2*, based on their successive requirements at distinct stages in the lineage progression of RPCs. We find further that LHX2 is required for the

normal response of RPCs to Sonic Hedgehog (SHH), a negative-feedback signal secreted by RGCs and known to limit their further generation. In addition, we show that both LHX2 and SHH promote the expression of *Ascl1*, a gene expressed in RPCs and implicated as molecular readout of competence progression. Together, the results presented here demonstrate how intrinsic factors such as LHX2 may perform distinct functions at distinct phases of RPC lineage progression to orchestrate the process of retinal neurogenesis.

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CHAPTER 1

INTRODUCTION

Early eye development

The eye is an intricate and complex organ whose development can be split into two major stages. During the first stage, patterning events partition the developing forebrain into separate regions, including that which will give rise to the eye; subsequent growth and morphogenesis then leads to formation of the familiar optic cup (OC) structure. In the second stage, the regionalization, growth, and differentiation of the OC and surrounding tissues generates a functional organ. A great deal is known about each of these developmental stages: here, we review this knowledge. It should be kept in mind that species-specific differences do exist, and while it is impossible to cover all of them, an effort has been made to explicitly acknowledge the most prominent instances.

Formation of the eye field

The first step in visual system development is specification of the eye field within the anterior neural plate, which occurs via the cooperative action of multiple eye-field transcription factors (EFTFs) – namely, *Rx* (*Rax*), *Six3*, *Six6* (*Optx2*), *Pax6*, *Lhx2*, *ET*, and *tll* (Zuber et al., 2003). Many positive regulatory interactions exist between these factors, and as a result, their overexpression (either together or in combination) is capable of generating ectopic eye structures (Chow et al., 1999; Loosli et al., 1999; Chuang and Raymond, 2001; Zuber et al., 2003). While generally associated with specification and proliferation during these and subsequent stages, the requirement for each gene varies and is revealed in part by the mutant phenotypes observed upon their loss, which range from complete absence of any eye structures, for *Six3* and *Rx* (Mathers et al., 1997; Carl et al., 2002), to arrest at optic vesicle stages, for *Lhx2* and *Pax6* (Hill et al., 1991; Porter et al., 1997), and mild or no defects, such as retinal hypoplasia, for *Six6* and *tll*

(Monaghan et al., 1997; Li et al., 2002).

After specification, the eye field is split by the anterior migration of diencephalic precursor cells (Varga et al., 1999; Hirose et al., 2004; England et al., 2006). This process is associated with Wnt11 (Heisenberg et al., 2000), which is necessary for the proper migration of these diencephalic cells (England et al., 2006), and Nodal (Rebagliati et al., 1998; Sampath et al., 1998), which is required for patterning, and the induction of Sonic Hedgehog (Shh) expression (Müller et al., 2000). In turn, the loss of Shh is associated with cyclopia (Chiang et al., 1996), which occurs when the eye field is not split correctly and thus only generates a single eye. Following this splitting of the eye field, visual system development proceeds with a complex series of three-dimensional morphogenetic movements, many of which have only recently been well-characterized, using advances in live-imaging and automated cell tracking (Keller et al., 2008) applied to both medaka and zebrafish model systems (England et al., 2006; Rembold et al., 2006; Kwan et al., 2012).

Evagination of the optic vesicles

At the beginning of neurulation, the eye field is bordered both anteriorly and laterally by the prospective telecephalon, and posteriorly by the prospective diencephalon. Forebrain cells begin to converge medially, and dorsolateral eye field cells follow, moving slower (Rembold et al., 2006; Brown et al., 2010). Though these dorsolateral eye field cells migrate, ventromedial eye field cells do not move significantly, leaving a wider neural tube at this point than is observed either anteriorly or posteriorly (Rembold et al., 2006). Diencephalic progenitor cells migrate both ventrally and anteriorly in bisecting the eye field, while telencephalic progenitors converge at the

dorsal midline (England et al., 2006). Eye field cells originally located in the dorsolateral aspect of the eye field switch direction upon reaching the midline (Rembold et al., 2006), and migrate ventrally and laterally to form optic sulci and eventually optic vesicles (OV) (Fig. 1.1A). Interestingly, proliferation does not seem to be required for initial evagination of the OV nor its subsequent growth (Harris and Hartenstein, 1991); rather, continued migration of individual cells into the OV may accounts for most, if not all, growth of the structure (Kwan et al., 2012). After formation of the OV, cells participate in a pinwheel movement as the entire structure elongates posteriorly before splitting into medial and lateral layers. Movement from the medial (presumptive retinal pigmented epithelium (pRPE)) to lateral (presumptive neural retina (pNR)) layers partially accounts for their differential growth in size (Kwan et al., 2012), coincident with the compaction and epithelialization of the OV, as well as early patterning events promoted by Fgf signaling (Picker et al., 2009). Again, the majority of these studies were performed in fish, and thus it remains unclear if similar mechanisms are utilized in higher vertebrates.

Invagination of the optic cup

After OV formation, the morphological transition to an OC structure occurs through epithelial folding, which is a common process observed in formation of other nervous system tissues, including the neural tube. This involves polarization of the neuroepithelial cells and, in some, a subsequent shape change from columnar to wedge-shaped, driven by asymmetric constriction. While this has predominantly been studied in the context of apical constriction (Sawyer et al., 2010), and is highly conserved, it is basal constriction which drives invagination of the OC (Martinez-Morales et al., 2009; Bogdanović et al., 2012). Coordinated with OC invagination is the specification and

invagination of the lens placode, which is derived from the overlying surface ectoderm (SE) (Fig. 1.1B). While FGF and BMP signals from the OV are necessary to induce lens formation (Furuta and Hogan, 1998; Faber et al., 2001; Yun et al., 2009), OC invagination requires a signal from the pre-lens ectoderm but not the lens placode itself (Hyer et al., 2003). In fact, recent studies in which mouse embryonic stem cells demonstrate the ability to form a self-organizing OC, invagination proceeds *in vitro*, despite the complete absence of overlying SE (Eiraku et al., 2011).

As growth and invagination of the OC proceed, they leave a temporary gap on the ventral side referred to as the optic fissure (OF), allowing for entrance of the hyaloid vessels, which supply blood to the eye during embryonic stages. After this is complete, however, the growing edges of the OC fuse to form a continuous tissue, instead leaving a central opening referred to as the optic disc, through which retinal ganglion cell (RGC) axons will eventually exit the eye. Failure of this fissure to occur results in coloboma, a common developmental defect in humans (Gregory-Evans et al., 2004; Chang et al., 2006) and one that has been associated with many different signaling pathways, including retinoic acid (RA) (Lupo et al., 2011), BMP (Morcillo et al., 2006), FGF (Cai et al., 2013a; 2014), WNT (Liu et al., 2012), and SHH (Morcillo et al., 2006; Zhang et al., 2009). These signals also play important roles in patterning the OC, and as a result, coloboma may arise as secondary defect – suggested by the observation that mutations in patterning genes downstream of these signals also lead to coloboma (Scholtz and Chan, 1987; Torres et al., 1996; Bertuzzi et al., 1999; Barbieri et al., 2002).

Patterning and regionalization

Coincident with invagination, axial patterning and regionalization of the OC are achieved through the combined influence of multiple signaling pathways (Fig. 1.1C). The distal region of the OV, after contact with the pre-lens SE, develops into pNR and is marked by expression of *Vsx2* (also known as *Chx10*), a process dependent on FGF signaling from the SE (Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Zhao et al., 2001). This simultaneously restricts expression of *Mitf*, which is initially expressed throughout the OV (Nguyen and Arnheiter, 2000), to the dorsal and proximal region that corresponds to pRPE. Similar to the FGF-mediated regulation of *Vsx2*, expression of RPE specific genes such as *Mitf* also depends to some extent on extraocular signals (Fuhrmann et al., 2000) such as WNT/β-catenin signaling (Westenskow et al., 2009; 2010). Subsequently, the mutually exclusive expression of *Vsx2* and *Mitf* is necessary to maintain identity of the neural retina and RPE, respectively, with antagonism maintained through transcriptional regulation and potential protein interactions (Zou and Levine, 2012) and mutations in either gene resulting in transdifferentiation (Bumsted and Barnstable, 2000; Rowan et al., 2004; Horsford et al., 2005; Bharti et al., 2012).

Dorsoventral (DV) patterning of the developing OC is mediated by the opposing influence of BMP and SHH signals, respectively (Zhang and Yang, 2001a; Peters and Cepko, 2002; Behesti et al., 2006; Kobayashi et al., 2010; Zhao et al., 2010). BMP4 expressed in the dorsal retina drives expression of the dorsal marker *Tbx5* (Koshiba-Takeuchi et al., 2000) and SHH expressed in the ventral midline drives expression of the ventral markers *Pax2* (Macdonald et al., 1995), *Vax2*, and *Vax1* (Take-uchi et al., 2003).

These ventral markers, particularly *Pax2*, mark the developing optic stalk (Nornes et al., 1990) and are required for proper closure of the OF, as mentioned above. Importantly, these dorsal and ventral specific genes are also important for establishing expression gradients for the Eph family member genes *EphB2*, *EphB3*, *ephrinB1* and *ephrinB2*, themselves in turn required for the correct projection of RGC axons and consequent formation the retinotopic map (Schulte et al., 1999; Koshiba-Takeuchi et al., 2000). Patterning along the anterior-posterior (AP, also referred to as nasal-temporal) axis of the retina is also important for the correct formation of retinotopic projections, though less is known about this process than DV patterning. Expression of the transcription factors *brain factor (BF) 1* and *2* (also known as *Foxg1* and *Foxd1*) is restricted to the anterior and posterior halves of the retina, respectively (Hatini et al., 1994; Yuasa et al., 1996). Subsequently to their expression, two additional transcription factors serve to mark the anterior retina, *SOHo-1* and *Gh6* (Deitcher et al., 1994; Schulte and Cepko, 2000). While relatively little is known about the upstream factors that dictate expression of these genes, AP and DV patterning may be somewhat related, as SHH signaling is affected in BF1 mutant mice (Huh et al., 1999).

At the conclusion of early eye development, distinct ocular structures (i.e., retina, RPE, lens, optic stalk) are set apart and patterned, with each subsequently developing in a relatively independent fashion. While we are principally concerned with the retina (and in particular the process of retinal neurogenesis), this overview of early eye provides a necessary context and perspective. Similarly, the following summary of *Lhx2* and its many context-dependent functions offers a background against which to interpret our results in the retina.

Functions of *Lhx2* during development

LIM-homeodomain (LIM-HD) transcription factors contain a homeodomain which facilitates DNA-binding and two LIM domains, specialized zinc fingers that allow for protein-protein interactions (Kadrmas and Beckerle, 2004). This presence of additional protein interaction domains is rare among homeodomain transcription factors (Hobert and Westphal, 2000), and results in a situation where multiple functions can be dictated through the interaction with various other proteins. LIM-homeobox (LHX) and LIM-only (LMO) proteins, both found exclusively in the nucleus, are capable of interacting with other transcription factors (Bach et al., 1997). In addition, their interaction with the obligate co-factor LIM-domain binding protein-1 (LDB1, also known as NLI), which itself is capable of dimerization, allows for the formation of heteromeric and homomeric complexes composed of different LIM proteins (Jurata et al., 1998). This provides a theoretical basis to explain how overlapping expression of these factors is capable of generating a code which can dictate cell fate within the spinal cord (Tsuchida et al., 1994). While often presumed to mediate transcriptional activation, LIM factors are also capable of binding RLIM, which recruits a transcriptional repressor complex (Bach et al., 1999). Thus, it is probable that tissue-specific interactions with different transcriptional regulators, as well as the ability to both positively and negatively regulate transcription, lead to the various roles of *Lhx2* during development.

Six families of LIM-HD proteins exist, each with typically a single member in invertebrates (such as *Drosophila melanogaster* or *C. elegans*) and two or more members in vertebrates (such as mice). *Lhx2* and *Lhx9* are the murine orthologs of *Drosophila apterous* and *C. elegans ttx-3* (Hobert and Westphal, 2000). While evolutionary distance

has allowed for some divergence, *apterous* and *Lhx2* display many similar traits, including widespread expression and the regulation of patterning and regional fate (Hobert and Westphal, 2000). In subsequent chapters, we describe the role of *Lhx2* in contributing to various properties of retinal progenitor cells during neurogenic stages. Here, however, we present the other, varied roles of *Lhx2*, in order to properly interpret and contextualize our results.

Roles in early eye development

Lhx2 is expressed from very early stages of eye development in many different model systems (Porter et al., 1997; Zuber et al., 2003; Seth et al., 2006; Viczian et al., 2006; Tétreault et al., 2009; Hägglund et al., 2011). As mentioned previously, it is one of several EFTFs whose combinatorial expression specifies the eye field in the anterior neural plate (Zuber et al., 2003). Displaying somewhat reciprocal regulation, *Lhx2* is required for the proper expression of other EFTFs (Tétreault et al., 2009), yet these factors are also capable of driving expression of *Lhx2* (Zuber et al., 2003). In spite of this, eye field specification and OV morphogenesis proceed normally in the absence of *Lhx2* (Yun et al., 2009). It is only at the subsequent OV-to-OC transition that *Lhx2* becomes absolutely required (Porter et al., 1997; Yun et al., 2009), with eye development arresting in both germline (Porter et al., 1997) and conditional knock-out (CKO) mutants (Hägglund et al., 2011). While these mice present with anophthalmia (Porter et al., 1997), *Lhx2* mutations are not a frequent cause of anophthalmia or mircophthalmia in humans (Desmaison et al., 2010), as such cases are likely missed due to the fact that a loss of *Lhx2* function would result in other developmental defects expected to compromise the viability of the fetus.

Previous work in our laboratory has shown that this arrest at the OV-to-OC transition is due to multiple functions for *Lhx2* in regulating lens induction, DV patterning, and regionalization (Yun et al., 2009). Interestingly, *Lhx2* seems to facilitate these processes through multiple mechanisms: the production of extracellular ligands (BMP4 and BMP7), the coupling of signaling pathways (SHH and/or FGF) with their context specific readouts (*Pax2* and *Vax2*), and the cell autonomous regulation of gene expression (*Vsx2* and *Mitf*) (Yun et al., 2009). These functions reveal the diverse regulatory capability of *Lhx2*, and though they may seem disparate, are supported by studies in other tissues that reveal a consistent theme – the selection or promotion of regional and/or cell type identity through support of correct gene expression patterns or programs.

Roles in other tissues

Hematopoietic system. *Lhx2* plays a critical role in the developing hematopoietic system, evidenced by the fact that *Lhx2*-null mice die *in utero* due to severe anemia (Porter et al., 1997). As the site of definitive erythropoiesis shifts to the fetal liver at approximately E12.0, erythrocyte production falters in *Lhx2*-null mice, and a low hematocrit (red blood cell count) is observed. A presumed defect in the establishment or proliferation of hematopoietic stem cells (HSCs) was rescued by transplanting *Lhx2*$^{-/-}$ fetal liver cells into wild-type hosts, and suggested that *Lhx2* was not required to support their formation in a cell-autonomous manner, but through non-cell autonomous mechanisms required for development of a permissive environment (Porter et al., 1997). Subsequent studies utilized this observation to generate HSC-like cell lines by overexpressing *Lhx2* in hematopoietic progenitors derived from embryonic stem cells

(Pinto do O et al., 1998; Kitajima et al., 2011), induced pluripotent stem cells (Kitajima et al., 2011), and primary cells - hematopoietic progenitors taken directly from adult bone marrow (Pinto do O et al., 2002). These HSC-like cell lines were found to renew through nonautonomous mechanisms (Pinto do O et al., 2001; Dahl et al., 2008), similar to findings in the *Lhx2*-null mutant and underscoring the role of *Lhx2* in regulating the expression of environmental factors (e.g., cell adhesion molecules, signaling factors, etc.)

Limb. *Lhx2* is expressed in the developing limb bud of vertebrates (Nohno et al., 1997; Rodriguez-Esteban et al., 1998; Rincon-Limas et al., 1999) just as its *Drosophila* ortholog *apterous* is expressed in the wing. However, while *apterous* is necessary for both dorsal-ventral patterning as well as outgrowth (Blair, 1993; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993), it appears that these functions have been delegated to separate, orthologous LIM factors in vertebrate development – *Lmx1* and *Lhx2*, respectively (Rodriguez-Esteban et al., 1998). While early studies of *Lhx2*-null mice did not reveal a clear phenotype in the developing limb (Porter et al., 1997), this was most likely due to redundancy with *Lhx9* (Tzchori et al., 2009). Accordingly, *Lhx2*, *Lhx9*, and *Lmx1b* are necessary for the facilitation of multiple signaling events and feedback loops in the developing limb, including the FGF8/FGF10 feedback loop required for proximal-distal growth and the expression of both SHH itself and SHH-target genes (notably *Grem1*, *Fgf4*, and *Bmp4*), necessary for maintenance of both the apical ectodermal ridge and zone of polarizing activity (Tzchori et al., 2009). Interestingly, the loss of all three LIM factors did not preclude expression of all *Shh*-responsive genes, as *Ptch1* was still expressed normally in their absence (Tzchori et al., 2009). Together with roles in both the OV and the hematopoietic lineage, these functions again emphasize a common function

for *Lhx2* in facilitating the expression of, and response to, extracellular signals.

Cortex and hippocampus. *Lhx2* is expressed in graded fashion along the developing cortex, with expression high in dorsomedial areas and low in both lateral and ventral areas, a pattern established by roof plate-derived BMP2 and BMP4 (Monuki et al., 2001). Within a critical window (E8.5-E10.5), *Lhx2* acts as a selector gene, necessary to allow specification of both the neocortex and hippocampal field – both of which are consequently absent in *Lhx2*-null mice (Porter et al., 1997; Bulchand et al., 2001) and replaced by a corresponding expansion of both the medial hem and lateral antihem fates (Mangale et al., 2008). The hem normally serves to organize the developing hippocampus by secreting both WNT and BMP signals, yet this expanded hem fails to induce hippocampal formation in these mice, demonstrating that *Lhx2* is required cell-autonomously, and downstream of these signals, to allow for this process. Accordingly, experiments done with chimeric mice reveal that ectopic hippocampal fields do form in patches of wild-type cells found adjacent to patches of $Lhx2^{-/-}$ cells – presumed hem (Mangale et al., 2008). Recently, it has been proposed that *Lhx2* functions in the same critical window (E8.5-E10.5) to restrict the extent of other, similar forebrain tissues including the septum and thalamic eminence (Roy et al., 2013), which themselves may act as secondary organizers.

Subsequent to specification of the cortex and hippocampus, *Lhx2* is necessary for maintenance of their identity, as well as further patterning and differentiation. Though removal of *Lhx2* at E10.5 does not prevent formation of the cortex, it results in a misspecification of three-layer paleocortex (also known as olfactory cortex or piriform cortex) rather than typical six-layer neocortex (Chou et al., 2009). Removal at E11.5,

again, does not prevent formation of the cortex but yields more subtle defects in both arealization and cortical organization (Shetty et al., 2013). Thus as development proceeds, *Lhx2* promotes and maintains neocortical fate on an increasingly fine scale, at the expense of alternate fates. In line with this theme, *Lhx2* expression is required in progenitor cell populations, of both the cortex and hippocampus, to ensure that specific aspects of their neurogenic programs are executed faithfully.

Specifically, continued expression of *Lhx2* is required to ensure adequate amounts of neurogenesis, through positive regulation of the Notch pathway (Subramanian et al., 2011; Chou and O'Leary, 2013). Targeted gain- and loss-of-function using electroporation suggested that *Lhx2* was necessary and sufficient for suppressing gliogenesis in hippocampal, yet not cortical, progenitors (Subramanian et al., 2011). In contrast, conditional inactivation with *NestinCre* did reveal a role for *Lhx2* in cortical progenitor cells, resulting in premature exit from the cell cycle and increased neurogenesis (Chou and O'Leary, 2013). Both studies linked these phenotypes to defects in Notch signaling, proposing that *Lhx2* was required for proper expression of the Notch-regulated transcription factors *Nfia* and *Hes1*, respectively. Our results, presented in subsequent chapters, detail a similar yet more complex role for *Lhx2* in regulating neurogenesis in the retina, also linking some aspects of the phenotype to defective Notch signaling.

Olfactory system and thalamus. In the olfactory epithelium, *Lhx2* is important for the maturation of olfactory sensory neurons (OSNs), though apparently not required for the proliferation and/or maintenance of progenitor cells. Progenitor cell markers including *Ascl1* and *Ngn1* are expressed normally, yet late-stage differentiation markers,

along with odorant receptor (OR) genes, are almost completely absent (Hirota and Mombaerts, 2004; Kolterud et al., 2004; Hirota et al., 2007). This failure of differentiated cells to fully mature likely accounts for the increase in apoptosis observed in the absence of *Lhx2* (Hirota and Mombaerts, 2004; Kolterud et al., 2004). This function contrasts with the regulation of *progenitor* cell properties observed in cortical structures (both the hippocampus and neocortex). Further, almost the opposite effect is observed in postmitotic retinal cells, where the ectopic expression of *Lhx2* promotes cell death (Sanuki et al., 2011). These studies, considered together, provide a reminder of how presumed interaction of *Lhx2* with different cell- and tissue-specific factors can lead to opposing and unexpected roles.

In addition to the failed maturation of OSNs, the olfactory systems of both germline and conditional *Lhx2* mutant mice display additional defects that introduce other important functions of the gene. Each OSN normally expresses a single OR gene, and all OSNs which express the same OR gene project to a specific glomerulus located within the olfactory bulb (OB). Within these glomeruli, OSNs synapse onto mitral cells of the OB, which themselves project along the lateral olfactory tract (LOT) to the olfactory cortex. Conditional inactivation of *Lhx2* in OSNs, in addition to affecting their maturation, results in a lack of innervation of the OB (Berghard et al., 2012) with OSN projections seemingly unable to reach their target, despite the continued presence of an olfactory bulb-like structure (Saha et al., 2007). Further, the mitral cells which compose the OB are unable to pioneer the LOT in *Lhx2*-null mice (Saha et al., 2007). While LIM domain proteins are heavily associated with the actin cytoskeleton (Kadrmas and Beckerle, 2004) and may be hypothesized to affect axon outgrowth in quite a direct

fashion, it must be remembered that both LHX and LMO factors are confined to the nucleus, and thus likely regulate the process indirectly. Often, transcription factors act as terminal selector genes, responsible for ensuring many properties of a differentiated cell (Hobert, 2011) - one relevant and well-described example is the regulation of AIY interneuron fate by transcription factors *ttx-3* (an *Lhx2/9* ortholog) and *ceh-10* (a *Vsx2* ortholog) in *C. elegans* (Bertrand and Hobert, 2009). In certain vertebrate tissues, *Lhx2* may similarly regulate the expression of genes necessary to confer cell-specific traits. This could include axon guidance receptors that ultimately determine connectivity, as different studies have reported defective axon guidance in the thalamus, spinal cord, and forebrain of *Lhx2* mutants, proposing or detailing roles for *Lhx2* in the regulation of *Robo* family receptors (Seth et al., 2006; Lakhina et al., 2007; Wilson et al., 2008; Chatterjee et al., 2012; Marcos-Mondéjar et al., 2012). These are reminiscent of the path-finding defects observed in the olfactory system of *Lhx2* mutants, and emphasize the varied roles of *Lhx2*.

Hair follicle. *Lhx2* is expressed from early stages of hair follicle morphogenesis (Rhee et al., 2006; Törnqvist et al., 2010) and maintained in hair follicle stem cells (HF-SCs) located in the bulge of the postnatal hair follicle (Rhee et al., 2006). There, *Lhx2* positively regulates the expression of the stem cell markers *Sox9*, *Tcf4*, and *Lgr5* (Mardaryev et al., 2011), and is necessary to suppress the precocious activation of HF-SCs and entry into an active growth phase, also known as anagen (Rhee et al., 2006; Mardaryev et al., 2011; Folgueras et al., 2013). This is reminiscent of the proposed role for *Lhx2* in suppressing reactivity and maintaining quiescence of Müller glia in the adult retina (de Melo et al., 2012), cells capable, in limited contexts, of mounting a

regenerative response to injury that may include the replacement of retinal neurons (Fischer and Reh, 2001; Raymond et al., 2006; Bernardos et al., 2007; Thummel et al., 2008; Ramachandran et al., 2010; Pollak et al., 2013). In both HF-SCs as well as Müller glia, these proposed roles in maintaining quiescence provide yet another contrast with the functions of *Lhx2* in 1) progenitor cell proliferation and 2) postmitotic cell maturation, described above. Interestingly, *Lhx2* may also be necessary for helping to maintain the architecture of the HF-SC niche by regulating many cytoskeletal and adhesion-related genes (Folgueras et al., 2013), similar to the known regulation of integrin expression in *Drosophila* by the ortholog *apterous* (Blair et al., 1994). This underscores the role of *Lhx2* in regulating axon growth and guidance as well as selective adhesion and tissue boundaries.

Many of the themes touched on here are reflected in our work on the function of *Lhx2* in RPCs. Before detailing these, however, we move from a generalized discussion of both eye development and *Lhx2* function to a more detailed description of the process at hand: retinal neurogenesis.

Later eye development and retinal histogenesis

After formation of the eye field, optic neuroepithelial cells undergo symmetric proliferative divisions to expand the size of the tissue. Though this proliferation is not required for OC morphogenesis (Harris and Hartenstein, 1991; Kwan et al., 2012), treatment with mitotic inhibitors does result in a significant reduction in size of the tissue. EFTFs are required to positively affect cell cycle progression during this time and, in turn, help dictate the size of resulting OV and OC structures (Mathers et al., 1997; Bernier et al., 2000; Loosli et al., 2001; Gestri et al., 2005; Tétreault et al., 2009). After

the expansion and morphogenesis of the tissue, it is the patterning and regionalization of the OC that ultimately sets apart, or creates, a distinct population of retinal progenitor cells (RPCs). Shortly after, retinal neurogenesis commences, propagating as a wave with RPCs beginning to produce postmitotic progeny and collectively switching to a balance between symmetric proliferative (self-expanding), asymmetric (self-renewing), and symmetric neurogenic (self-consuming) divisions. How the initiation and propagation of this wave are regulated represent important and open questions, with different lines of evidence implicating both intrinsic and extrinsic factors in each process.

Initiation of neurogenesis

Though several intrinsic factors affect the timing of neurogenesis, a unified picture of their function or mechanism is somewhat lacking. One of the most well-studied factors in RPCs is *Vsx2*, which is thought to be the most accurate and specific marker of RPCs. It is necessary not only for the specification and maintenance of retinal identity, but also proper levels of proliferation and the timely initiation of neurogenesis. In *Vsx2*-null mice (also referred to as *ocular retardation* or *orJ*), neurogenesis is delayed by one to two days (Bone-Larson et al., 2000; Sigulinsky et al., 2008). Recent work in our laboratory using genetic chimeras has revealed that this defect is cell-autonomous, with *Vsx2*-negative RPCs failing to differentiate on the proper schedule even in the presence of neighboring, wild-type RPCs (Sigulinsky et al., unpublished). While the mechanism regulating this phenotype remains unclear, previous work in our laboratory has also linked *Vsx2* with intrinsic cell-cycle regulators. Proliferation is greatly reduced in *Vsx2*-null mice and yields a hypocellular retina (Burmeister et al., 1996; Sigulinsky et al., 2008), which can be rescued by additional deletion of the cyclin-dependent kinase

inhibitor (CDKI) *p27^{Kip1}* (Green et al., 2003). Expression of *p27^{Kip1}* is normally elevated in the *Vsx2*-null retina due to a loss of post-transcriptional regulation by Cyclin D1 (Ccnd1), itself requiring *Vsx2* for proper expression and providing a possible connection. In regards to controlling the initiation of neurogenesis, this observation is interesting in that another large body of evidence, outside of the retina, suggests that the accumulation of *p27^{Kip1}* may act as a cell-intrinsic timer that drives cell-cycle exit (Temple and Raff, 1986; Hughes and Raff, 1987; Friessen et al., 1997; Gao et al., 1997; Tikoo et al., 1997; Durand et al., 1998; Gao et al., 1998; Durand and Raff, 2000). However, it is unlikely that such a mechanism, by itself, is responsible for initiating neurogenesis. It must be remembered that progression through the cell-cycle is not required for neurogenesis in the retina (Harris and Hartenstein, 1991). And further, several of these studies also implicate the influence of extrinsic factors (Durand et al., 1998; Gao et al., 1998; Durand and Raff, 2000).

Additional intrinsic factors associated with the initiation of neurogenesis are *Pax6* and *Sox2*, whose expression levels are important for determining retinal identity (Taranova et al., 2006; Matsushima et al., 2011). Subsequent to retinal specification, *Pax6* and *Sox2* are both required for the expression of the proneural bHLH transcription factor *Atoh7* (also known as *Math5*) (Brown et al., 1998; Taranova et al., 2006; Willardsen et al., 2009; Riesenberg et al., 2009a), which is required for generation of the first-born neuronal cell type, RGCs (Brown et al., 1998; 2001). In addition, removal of *Pax6* precludes expression of other proneural bHLH genes, including *Ngn2* and *Ascl1* (also known as *Mash1*) (Marquardt et al., 2001). *Ngn2* is the first of these proneural bHLH genes to be expressed in the retina following the downregulation of *Pax2*

(Hufnagel et al., 2010), and the restriction of *Pax2* to the presumptive optic stalk (Nornes et al., 1990) allows an expansion of *Pax6* expression that helps to define the presumptive retina and allow for *Ngn2* expression (Schwarz et al., 2000). It is fair to reason that the expression levels of these genes (*Vsx2*, *Sox2*, *Pax6*, etc.) may, in part, dictate the timing of neurogenesis. Yet, there is evidence demonstrating that their expression is regulated by extrinsic signals (Macdonald et al., 1995; Van Raay et al., 2005; Agathocleous et al., 2009; Cai et al., 2013b), making it difficult to separate the influence of intrinsic and extrinsic factors.

Many different extrinsic signals regulate neurogenesis by affecting gene expression and/or cell type production at later stages of the process, yet few signaling pathways have been implicated in directly regulating the initiation of neurogenesis. Given its association with retinal induction (Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Zhao et al., 2001), it is perhaps not surprising that FGF signaling is the most strongly linked to initiation of neurogenesis. In both zebrafish and chicks, FGFs drive neurogenesis and promote expression of *Atoh7* homologs *Cath5* and *ath5* (McCabe et al., 1999; Martinez-Morales et al., 2005; McCabe et al., 2006). SHH, derived from newborn neuronal cells, is heavily implicated in the spread of the neurogenic wave (discussed below). However, at least one study suggests that midline SHH, responsible for patterning the OC (discussed previously), may also regulate the initiation of neurogenesis by setting an intrinsic timer within RPCs (Kay et al., 2005). Subsequent to initiation, however, the situation is somewhat reversed in that the influence of extrinsic signals has received more attention in propagating the wave than intrinsic factors.

Propagation of the neurogenic wave

While there is some anatomical variation between species in terms of the location of initiation, neurogenesis spreads throughout the retina as a wave after initiating in a discrete domain. In *Drosophila*, this wave of differentiation is referred to as the morphogenetic furrow (MF), and sweeps from posterior to anterior across the eye imaginal disc (Heberlein and Moses, 1995); waves of differentiation also pattern the developing lamina and medulla, other structures of the *Drosophila* visual system found in the optic lobe (Sato et al., 2007; Egger et al., 2010). In zebrafish, neurogenesis begins in the ventro-nasal retina before spreading around the optic stalk in a fan pattern (Hu and Easter, 1999) and in the chick retina, neurogenesis begins in a dorso-temporal region near the central retina before spreading to the periphery (Prada et al., 1991). Similar to this, neurogenesis initiates in the dorso-central region of the developing mouse retina (Fig. 1.2) (Hufnagel et al., 2010). Many different studies have demonstrated that, similar to the initiation of neurogenesis, correct propagation of these waves requires the coordinated action of both intrinsic and extrinsic factors.

Recent work in the mouse retina demonstrated that *Ngn2* is essential for propagation of the neurogenic wave, as the spread is stalled quickly after initiation in mice lacking *Ngn2* (Hufnagel et al., 2010). However, spread of the wave is restored after approximately two days; this is due to *Ascl1*, which is normally expressed a few days after *Ngn2* and not only responsible for activating a second wave of differentiation (Hufnagel et al., 2010). The exact mechanism(s) by which *Ngn2*, and subsequently *Ascl1*, promote wave progression remains unclear, yet support for such a function is found in the similar role of *Drosophila atonal* - orthologous to *Ngn2* and required for progression of

the MF in the eye imaginal disc (Jarman et al., 1995). Despite the lack of a clear mechanism, other lines of evidence indicate that the sustained and/or threshold expression of these proneural bHLH transcription factors is a common prerequisite for differentiation in the vertebrate CNS (Shimojo et al., 2008; Imayoshi et al., 2010). Their expression is closely tied to activity of Notch signaling, however, and therefore linked to extrinsic influence, just as with the potential influence of *Sox2* and *Pax6* in neurogenesis initiation.

Multiple signaling pathways influence the progression of the neurogenic wavefront, most notably Notch and Hedgehog. Each is reviewed within subsequent sections of this chapter, yet their potential roles in this process are briefly covered here. Notch signaling, while well-known to work through lateral inhibition and limit the rate and extent of differentiation during neurogenesis (i.e., behind the wave), may also play a role in regulating the progression of the wavefront. In both mice and chicks, *Delta-like 1* (*Dll1*), one of several vertebrate Notch ligands, is expressed in RPCs (Nelson and Reh, 2008; Nelson et al., 2009). Furthermore, this expression occurs ahead of the neurogenic wave (Rocha et al., 2009), and in the *Drosophila* optic lobe, *Delta* is also expressed at high levels in front of the wave of differentiation (Egger et al., 2010). It is thought that after cells acquire neurogenic potential with passing of the neurogenic wavefront, they depend on lateral inhibition to maintain their progenitor status. Thus, high levels of *Dll1* expression at or ahead of the neurogenic wavefront are postulated to prevent the immediate differentiation of newly neurogenic cells, as Notch ligands are not expressed prior to neurogenesis (Nelson et al., 2009; Rocha et al., 2009) and bordering, non-neurogenic cells would be incapable of providing inhibition, if not for this elevation in

Dll1 expression (Formosa-Jordan et al., 2012). Such a role in slowing of the wavefront is consistent with the observation that cells in the center of *Delta* expressing clones are prevented from differentiating, presumably through a mechanism of mutual inhibition (Dorsky et al., 1997). However, to truly separate potential roles in regulating the amount vs. the timing of differentiation will require closer examination of wavefront progression in *Dll1* mutant mice. Thus, defining a clear role for Notch signaling in this process still requires further study.

While the proposed role of Hedgehog in promoting wave propagation is relatively well articulated within the literature, experimental evidence is somewhat conflicting. In *Drosophila*, *hedgehog* is expressed by newly differentiated photoreceptors behind the MF, and in vertebrates, the ortholog *Shh* is expressed by newly formed RGCs, the first born neuronal cell type. Studies in both *Drosophila* and zebrafish have proposed that hedgehog and SHH, respectively, diffuse across the wavefront to drive further differentiation (and thus their own expression) in neighboring cells, effectively propagating the wave (Heberlein et al., 1993; Ma et al., 1993; Masai et al., 2000; Neumann and Nuesslein-Volhard, 2000; Masai et al., 2005). Yet at least one report, mentioned above, suggests that RPCs appear prespecified to differentiate at given times, with SHH acting to help set this timer (Kay et al., 2005). Extensive work in the murine retina also showed that rather than driving cell-cycle exit, and thus the wave of differentiation, SHH is responsible for driving the continued proliferation of RPCs (Jensen and Wallace, 1997; Levine et al., 1997; Moshiri and Reh, 2004; Moshiri et al., 2005; Wang et al., 2005). Thus, despite some similarities, mechanisms for propagation are likely distinct in Drosophila and vertebrates, with the observed species-specific

differences between mice and zebrafish likely reconciled through a model in which SHH promotes both faster progression through the cell-cycle as well as a commitment toward differentiation (Locker et al., 2006; Agathocleous et al., 2007).

As RPCs do begin to differentiate they produce a defined set of cell types, and genetic manipulation of many different factors, over the past two decades, is beginning to generate an understanding of their roles in fate regulation. While environmental signals no doubt continue to play a large role in the process, the following chapter will focus mainly on these intrinsic factors.

Timing and generation of the different retinal cell classes

Classical lineage tracing experiments demonstrated that RPCs are multipotent, capable of giving rise to all seven major cell types of the adult retina (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). And birth-dating studies have shown that these cell types are produced in a distinct yet overlapping order (Fig. 1.3) (Carter-Dawson and LaVail, 1979; Young, 1985; Harman and Beazley, 1989; Rapaport et al., 2004; Wong and Rapaport, 2009), though small differences are observed between species. In the murine retina, RGCs are produced first, followed closely by cone photoreceptors, horizontal cells, and amacrine cells; rod photoreceptors are produced next, followed by bipolar cells and finally Müller glia (Rapaport et al., 2004). In the adult retina, these cell types reside in distinct layers (Fig. 1.4): the outer nuclear layer (ONL) contains both rod and cone photoreceptors, the inner nuclear layer (INL) contains Müller glia as well as bipolar, horizontal, and amacrine cells, and the ganglion cell layer (GCL) contains both RGCs as well as displaced amacrine cells. In forming connections, the processes of retinal neurons are primarily confined to two additional layers: the outer

plexiform layer (OPL), which separates the ONL and INL, and the inner plexiform layer (IPL), which separates the INL and GCL. While many of these different cell types can be further divided into numerous subtypes on the basis of morphology, function, and gene expression, we will focus here mainly on the major cell types as listed above, briefly introducing their function and reviewing what is known regarding the mechanisms of their generation.

RGCs. RGCs are the first-born cell type in the developing retina, and their appearance coincides with the beginning of neurogenesis, at approximately E11.5-E12.0 in the mouse (Hufnagel et al., 2010). Subsequent to development, they represent the sole output of the retina, as their axons form the optic nerve, projecting to regions such as the superior colliculus and lateral geniculate nucleus of the thalamus. Their genesis is strongly associated with the proneural bHLH transcription factor *Atoh7* and its orthologs (Kanekar et al., 1997; Brown et al., 1998; Masai et al., 2000; Matter-Sadzinski et al., 2001). Similar to many other proneural bHLH factors which influence RPC fate choice, *Atoh7* is expressed either shortly before or quickly after cell-cycle exit (Brzezinski et al., 2012). While *Atoh7* is thought to be necessary for RGC genesis in mice (Brown et al., 2001), only ~10% of the lineage adopts the RGC fate (Brzezinski et al., 2012), and a small amount of RGCs do develop in its absence (Wang et al., 2001; Lin et al., 2004). This is potentially due to redundancy, as some bHLH factors (Mao et al., 2008b), yet not others (Hufnagel et al., 2013), can partially compensate for *Atoh7* when expressed from the endogenous locus. Further, *Atoh7* itself, when misexpressed, is not sufficient to confer the RGC fate (Prasov and Glaser, 2012). This serves as a prototypical example of fate regulation in the retina, in that while single factors are usually associated with or

seemingly required for the generation of certain cell types, it is the combined and redundant influence of many factors that likely dictates fate choice in RPCs. In the murine retina, there are approximately 25 different subtypes of RGCs (Völgyi et al., 2009; Kim et al., 2010; Sanes and Zipursky, 2010; Kay et al., 2011a), and notably, RGCs are one major cell type for which some progress has recently been made in identifying potential mechanisms responsible for subtype generation (la Huerta et al., 2012). Downstream of their specification, further, many different studies have given us a good understanding of the gene network required for the further differentiation and maturation of RGCs, which includes the *Pou4f* (also known as *Brn3*) family of factors (Badea et al., 2009; Shi et al., 2013), *Isl1* (Mu et al., 2008; Pan et al., 2008), and *Eomes* (Mao et al., 2008a).

Amacrine and horizontal cells. Closely following the genesis of RGCs, both horizontal cells and amacrine cells are generated in the embryonic retina. While they represent distinct classes of cells, their specification is controlled in part through common mechanisms (Poché and Reese, 2009); thus, they are considered together here. Horizontal cells occupy the outer-most aspect of the INL (adjacent to the OPL) and modify signaling between photoreceptors and bipolar cells. Amacrine cells, found in the inner-most aspect of the INL (adjacent to the IPL) as well as the GCL, modify the transmission of information between bipolar cells and RGCs. Thus, both cell types serve as retinal interneurons. The proneural bHLH factors *Neurod* (also known as *NeuroD1*) and *Math3* (also known as *NeuroD4*) are closely associated with amacrine cell genesis, and each is expressed transiently by differentiating amacrine cells (Morrow et al., 1999; Inoue et al., 2002). Loss of either factor individually does not affect amacrine cell number, but

Neurod$^{-/-}$*Math3*$^{-/-}$ double mutants show almost a complete lack of amacrine cells (Inoue et al., 2002). This is accompanied by an increase in both the expression of *Atoh7*, the number of RGCs, and the number of Müller glia (Inoue et al., 2002), suggesting that antagonism between competing bHLH factors plays an important role in cell fate decisions. The overexpression of either factor alone promotes the production of rods, while misexpression of *Neurod* or *Math3*, together with either *Pax6* or *Six3,* promotes amacrine or both amacrine and horizontal cell fates, respectively (Inoue et al., 2002). This apparent redundancy in function between *Pax6* and *Six3* may explain the exclusive production of amacrine cells seen in the *Pax6* conditional mutant (Marquardt et al., 2001). In addition to these factors, *Foxn4* and *Ptf1a*, expressed in RPCs and postmitotic precursors, respectively (Li et al., 2004; Fujitani et al., 2006), play important roles in amacrine and horizontal cell genesis. Loss of either factor results in the complete loss of horizontal cells and near-complete loss of amacrine cells (Li et al., 2004; Fujitani et al., 2006; Nakhai et al., 2007). Based on expression patterns analyzed in mutant mice, *Ptf1a* is thought to act downstream of *Foxn4* and in parallel with *Neurod* and *Math3* (Li et al., 2004; Fujitani et al., 2006). Accordingly, the loss of *Ptf1a* (similar to the loss of *Neurod* and *Math3* together) also results in a reallocation, or specification, of these cells into the RGC fate (Fujitani et al., 2006). While overexpression of *Ptf1a* in this study (Fujitani et al., 2006) was not sufficient to promote horizontal or amacrine cell fates, similar experiments in zebrafish (Jusuf et al., 2011), *Xenopus* (Dullin et al., 2007), and chick (Lelièvre et al., 2011) report positive results – that ectopic expression does in fact promote the cell-autonomous acquisition of these fates. Loss of another downstream gene, *Prox1*, results in a complete lack of horizontal cells (Dyer et al., 2003). However,

as *Prox1* also promotes cell-cycle exit, no increases in other cell types were reported in the *Prox1-/-* mutant retina. Rather, an increase in proliferation is observed (Dyer et al., 2003). Together, the above studies have not only identified a clear set of factors which influence the genesis of horizontal and amacrine cells, but suggest a model in which early RPCs are biased toward the generation of RGCs and require additional, or antagonistic, influence for the generation of these interneuron cell types (Jusuf et al., 2011).

While there is only a single type of horizontal cell identified in mice (and only two to three in several other species) (Génis-Gálvez et al., 1981; Peichl and González-Soriano, 1994; Tanabe et al., 2006), amacrine cells represent the most diverse cell class, with over 30 recognized subtypes (MacNeil and Masland, 1998; MacNeil et al., 1999). In addition, the timing and rate of amacrine cell production displays characteristics usually associated with only early- or late-born cell types (Rapaport et al., 2004), suggesting that distinct amacrine cell subtypes may belong to one group or the other; this is supported by the observation that certain amacrine cell subtypes are in fact generated in a particular order (Cherry et al., 2009; Voinescu et al., 2009). Thus, the production of their subtypes deserves some brief consideration. *Neurod* and *Math3* have established roles in regulating the genesis of all amacrine cells, yet single cell profiling and functional studies have revealed that two other *NeuroD* family members, *NeuroD2* and *NeuroD6*, are expressed in specific amacrine cell subtypes and required for their generation (Cherry et al., 2011; Kay et al., 2011b). Somewhat similarly, while *Ptf1a* is required for the genesis of all horizontal and most amacrine cells (Fujitani et al., 2006; Nakhai et al., 2007), work in zebrafish has proposed that its combined expression with other lineage markers (such as *ath5*) serves to specify individual subtypes that arise from distinct cohorts of RPCs (Jusuf

et al., 2011), in part through regulating the expression of the homeobox transcription factor *Barhl2* (Jusuf et al., 2012). In line with this work, loss of *Barhl2* in the developing mouse retina significantly alters the relative proportion of amacrine cell subtypes (Ding et al., 2009). Finally, it should be noted that just as *Ptf1a* may act in concert with other factors to specify the fate of amacrine cells or subtypes thereof (Jusuf et al., 2011), the same may be true for specification of horizontal cells, as such a role in combined regulation was recently suggested for *Ptf1a* and *Onecut1* (*Oc1*) (Wu et al., 2013). And just as a certain biased RPCs may employ these mechanisms of combinatorial expression patterns to choose between amacrine and horizontal cell types and subtypes, other RPCs may similarly choose between the cone and rod photoreceptor fates; indeed, *Oc1* has also been suggested to play a role in that decision (Emerson et al., 2013).

Cone and rod photoreceptors. Cone and rod photoreceptors represent the sensory neurons of the visual system, hyperpolarizing in response to light and transmitting visual information to cells of the INL before it is ultimately passed to RGCs and sent to higher visual areas in the brain. In the murine retina, cone and rod photoreceptors are generated during early and late phases of neurogenesis, respectively, with the specification of a generalized photoreceptor fate linked to certain transcription factors and the subsequent decision to adopt either a rod or cone fate decided by others (Swaroop et al., 2010; Forrest and Swaroop, 2012). The homeobox transcription factor *Otx2* is the earliest known marker of photoreceptor fate, though Otx2+ RPCs also give rise to bipolar cells, and Otx2 may even be transiently expressed by other cell classes such as RGCs and amacrine cells (Bovolenta et al., 1997). Similar to *Atoh7*, *Otx2* is expressed in RPCs either during their terminal division or quickly after cell-cycle exit (Bovolenta et al.,

1997; Nishida et al., 2003). It is required for the expression of the *Crx* (Nishida et al., 2003), another homeobox transcription factor expressed specifically in developing and mature photoreceptors (Chen et al., 1997; Furukawa et al., 1997) that is necessary for the activation of many photoreceptor specific genes and the subsequent maturation of these cell types (Furukawa et al., 1999). Downstream of *Otx2* and *Crx*, *Nrl* is thought of as the first rod-specific marker in the neural retina, necessary for the determination of that cell type (Swaroop et al., 1992; Mears et al., 2001). The *Nrl*-mediated suppression of cone-specific gene programs is accomplished in part through promotion of the nuclear receptor *Nr2e3*, another gene whose expression is confined to postmitotic rod precursors (Chen et al., 2005; Peng et al., 2005; Oh et al., 2008). Reciprocal to the expression of *Nrl* and *Nr2e3*, recent evidence suggests that cones may be specified through the cooperative action of *Oc1* and *Otx2* (Emerson et al., 2013), with differentiation promoted through the activity of *Sall3* (de Melo et al., 2011) and another nuclear receptor, *Rorα* (Fujieda et al., 2009). The subsequent decision to express either M- or S-opsin is mediated by the nuclear receptor *TRβ2* (Ng et al., 2001; 2010; 2011).

Bipolar cells. Bipolar cells reside in the INL and serve to pass visual information from photoreceptors to RGCs. They are the last neuronal cell type to appear during retinal neurogenesis, produced postnatally in the murine retina in partial overlap with both rod photoreceptors and Müller glia (Rapaport et al., 2004). Similar to the role of *Neurod* and *Math3* in amacrine cell genesis, there is evidence that the cooperative expression of *Ascl1* and *Math3* is required for bipolar cell determination, as both are expressed transiently by differentiating bipolar cells (Jasoni and Reh, 1996; Roztocil et al., 1997). Their numbers are slightly reduced, or unaffected, in the respective single

mutations, yet they are completely missing in the double mutant (Tomita et al., 2000); in their place, the number of Müller glia is increased significantly. *Vsx2* is also required for bipolar cell development, as these cells are completely missing in the *orJ* mouse (Burmeister et al., 1996). Accordingly, while overexpression of *Vsx2*, *Mash1* or *Math3* alone does not drive bipolar cell formation, expression of either *Mash1* or *Math3* in combination with *Vsx2* does increase their number (Hatakeyama et al., 2001). Again, similar to amacrine cell genesis, more recent work has supplemented our knowledge of the factors that regulate this decision, bringing to light the role of *Blimp1* (also known as *Prdm1*). Lineage tracing, conditional inactivation, and enhancer studies have revealed that *Blimp1* is expressed in all Otx2+ cells, preventing the formation of bipolar cells and stabilizing the rod photoreceptor fate through direct repression of both *Otx2* and *Vsx2* (Brzezinski et al., 2010; Katoh et al., 2010; Brzezinski et al., 2013; Wang et al., 2014a). This is in line with a previous study showing that overexpression of *Vsx2* promoted bipolar formation and loss of *Vsx2* promoted rod formation (Livne-bar, 2006). As *Blimp1* is expressed in many Otx2+ cells, it first appears in the embryonic retina at approximately E12.5 (Brzezinski et al., 2010; Katoh et al., 2010) and may even play a role in stabilizing the cone photoreceptor fate, as cones are lost in *Blimp1* conditional mutants. However, there is no reciprocal increase in other early-born cell types, and thus, *Blimp1* (at least currently) does not appear to regulate a binary fate decision among early progenitors, as it does in deciding the rod/bipolar cell decision.

Müller glia. Following the production of all six major neuronal cell types, RPCs give rise to the predominant glial cell type of the adult retina - Müller glia. These cells have diverse and species-specific functions in the retina, from mediating response to

injury and wound healing to the continued generation or regeneration of retinal neurons in the adult retina. Their cell bodies are located within the INL, and they have processes that span the entire apical-basal width of the retina. As they are the last and only non-neuronal cell type generated from RPCs, their production is associated with both *Hes1* and *Hes5*, which serve to maintain the RPC pool and prevent excess neurogenesis by mediating Notch signaling activity and antagonizing other proneural bHLH factors. *Hes5* is expressed in differentiating Müller glia, and loss or overexpression of *Hes5* results in a decrease and increase, respectively, in their number (Hojo et al., 2000). Similarly, *Hes1* is expressed in differentiating Müller glia, and misexpressing a dominant negative *Hes1* leads to a reduction in their number while overexpressing a wild-type *Hes1* leads to an increase in their number (Furukawa et al., 2000). The *Hes*-related gene family (also known as Hey/HRT/CHF/*gridlock*) is composed of three similar bHLH transcription factors, all of which are expressed in the developing retina. Of these, however, *Hey2* is the only one that seems to regulate Müller glia formation. *Hey2* is expressed in Müller glia, and overexpression drives the production of Müller glia, similar to both *Hes1* and *Hes5* (Satow et al., 2001). Overexpression of the receptor *Notch1* promotes a similar overproduction of Müller glia (Bao and Cepko, 1997; Furukawa et al., 2000; Scheer et al., 2001), and conditional inactivation leads to an underproduction of Müller glia (Jadhav et al., 2006; Yaron et al., 2006), among other defects. In addition to this heavy association with Müller glia, recent evidence has also pointed toward a role for Notch signaling in promoting the generation of both bipolar cells at the expense of more abundant rod photoreceptors (Kechad et al., 2012; Mizeracka et al., 2013a; 2013b).

In summary, a great deal is known about the individual factors that are necessary

and sufficient, either alone or in combination, for the generation of major retinal cell types. While this detailed level of knowledge regarding every single cell type is perhaps not required to simply understand our work, it is required to contextualize our results and allow us to form accurate and useful models that can direct and inform future research. In the same vein, we will now consider one additional aspect of this process - the factors that regulate or dictate the timing, or ordered production, of these cell types. Only a handful of studies have implicated different genes in the promotion or execution of competence changes, and we briefly touch on them here.

Competence progression

Early lineage tracing studies suggested that each RPC had the potential to generate all seven major cell types of the retina (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990), though it was initially unclear why these cell types arose in a characteristic order. Heterochronic cultures and transplant experiments subsequently demonstrated that embryonic RPCs could not be induced to prematurely generate later-born cell types when exposed to an older environment (Watanabe and Raff, 1990; Morrow et al., 1998; Rapaport et al., 2001), and that older, postnatal RPCs could not be induced to return to the generation of early-born cell types (Belliveau et al., 2000). These studies, together with others that implicated the influence of extrinsic signals (Reh and Tully, 1986; Altshuler and Cepko, 1992; Watanabe and Raff, 1992; Belliveau and Cepko, 1999), led to the formulation of the competence model, which proposes that each RPC transitions irreversibly through a series of intrinsically defined states, competent at any given moment to generate only a subset of retinal cell types in response to the appropriate environmental cues (Cepko et al., 1996). Multiple

studies have supported this hypothesis over the intervening years, and it remains the most prevalent model to date. However, several important issues remain unresolved, including 1) which intrinsic factors define and/or limit competence, 2) what intrinsic or extrinsic factors provide temporal cues to initiate these changes in competence and 3) what mechanisms are used to execute these changes.

Given that the competence progression of vertebrate RPCs mirrors that previously studied in *Drosophila* neuroblasts, it is perhaps unsurprising that similar mechanisms may play a role. In the ventral nerve cord of *Drosophila*, neuroblasts sequentially express a series of transcription factors that are inherited by their progeny and subsequently dictate the temporally-guided production of different cell types (Isshiki et al., 2001). The earliest of these factors, *Hunchback* (*Hb*), has an ortholog in mice, *Ikaros*, that has since been proposed to confer competence for early-born cell types in the both the retina (Elliott et al., 2008) and cortex (Alsiö et al., 2013). Whether additional, sequentially expressed factors can be identified that confer the competence to generate later-born cell types is an open question, complicated by the fact that their identity may not correspond directly to remaining orthologs of the *Drosophila* sequence (*Krüppel*, *Pdm*, and *Castor*); indeed, a completely separate set of factors has recently been found to serve the same function in a different location of the *Drosophila* CNS (Li et al., 2013). In terms of executing competence changes, the progression through both series of these transcription factors in *Drosophila* appears to depend on cross-regulatory interactions. However, there is now evidence for a different mechanism regulating competence progression in vertebrate RPCs.

Accumulating evidence suggests that micro-RNAs (miRNAs) are in part responsible for executing competence changes in the retina, though a connection to *Ikaros* has not been forthcoming. Studies in *Xenopus* first demonstrated that miRNAs were responsible for preventing the premature expression of *Xotx2* and *Xvsx1*, as well as the corresponding genesis of bipolar cells (for which both genes are required) (Decembrini et al., 2006; 2008; 2009). In mice, miRNAs also play a role in facilitating competence progression, yet in an inverse fashion. Rather than preventing premature expression of genes normally expressed at late stages (as with *Xotx2* and *Xvsx1*), they are required to prevent prolonged expression of genes normally expressed at early stages – *Protogenin* (*Prtg*) and *Lin28b* (Georgi and Reh, 2010; La Torre et al., 2013). While these two genes are therefore potentially similar to *Ikaros* in that they may confer competence to generate early-born cell types, re-expression of *Lin28b* at late stages of neurogenesis was not able to induce the production of early-born cell types (La Torre et al., 2013), in contrast to what was observed with *Ikaros* (Elliott et al., 2008). While a role for all three genes (*Ikaros*, *Prtg*, and *Lin28b*) in regulating competence progression is supported by studies outside the retina (Wong et al., 2010; Ambros, 2011; Alsiö et al., 2013), it is unclear at this time how they may or may not function together.

Competence is an intrinsic property, defined through the cell autonomous expression of a certain factor or combination of factors. And while progress has been made in both identifying these factors and determining how their expression is regulated (above), the question of how individual cells know *when* to transition between competence states remains largely unanswered. Extrinsic signals are excellent candidates to convey such information, potentially serving not to simultaneously end the production

of one cell type (through negative feedback) but signal production of the next (through feedforward regulation). While various early studies postulated the role of both negative (Reh and Tully, 1986; Waid and McLoon, 1998; Belliveau and Cepko, 1999) and positive feedback (Altshuler and Cepko, 1992; Watanabe and Raff, 1992) in the production of different cell types, few factors have actually been identified as having such a role. The most prominent are GDF11 and SHH, both secreted from newly-born RGCs and used to subsequently limit further production of RGCs (Zhang and Yang, 2001b; Kim, 2005; Wang et al., 2005). While only a single study has implicated GDF11 in this regard (Kim et al., 2005), such a role for SHH has also been corroborated by more recent work (Sakagami et al., 2009; Cwinn et al., 2011). Both are attractive candidates for mediating competence progression, yet defining any potential roles in that process will require further study. GDF11 was theorized to regulate competence progression, as its loss resulted in the reduced expression of several genes associated with production of later-born cell types – including *Ascl1* (Kim et al., 2005). While SHH has not been explicitly proposed to regulate competence, there is evidence that pathway inactivation artificially extends RGC production (as with GDF11) (Wang et al., 2005). In Chapter 4, we detail evidence from our own work that implies such a function for SHH.

Sonic hedgehog signaling

Background and pathway components

Genetic screens in *Drosophila* first identified *hedgehog* as a gene important for establishing segment polarity (Nüsslein-Volhard and Wieschaus, 1980), and there are three known orthologs in vertebrates: *Sonic hedgehog* (*Shh*), *Desert hedgehog* (*Dhh*), and *Indian hedgehog* (*Ihh*) (Echelard et al., 1993; Krauss et al., 1993; Chang et al., 1994;

Roelink et al., 1994). Both *Shh* and *Dhh* are expressed in the developing mouse retina, while *Ihh* expression is found in the RPE (Jensen and Wallace, 1997; Levine et al., 1997). Of these, *Shh* is the most prominent in eye development, with multiple roles during both early (discussed earlier) and late stages (discussed below). Before elaborating on these functions, however, it is necessary to give an overview of the pathway, and introduce the various steps required for signal transduction.

While the exact mechanisms regulating the release and spread of Hedgehog (HH) ligands remain active areas of investigation, it is clear that multiple post-translational modifications play an important role. After translation, all HH proteins undergo autoproteolytic cleavage, generating both a C-terminal and N-terminal peptide, the latter of which is modified via the addition of cholesterol to its carboxy-terminal end (Lee et al., 1992; 1994; Porter et al., 1995; 1996) and a palmitic acid group to its amino-terminal end (Chamoun et al., 2001). The resulting fragment is then transported to the plasma membrane and thought to be secreted via the combined action of the proteins Dispatched and Scube2 (Creanga et al., 2012; Tukachinsky et al., 2012). While only the cholesterol modifications are required for secretion, both cholesterol and palmitate modifications are required for multimerization of HH proteins outside of the cell, a process thought to potentiate the spread and long-range signaling of HH (Lewis et al., 2001; Zeng et al., 2001; Gallet et al., 2003; Chen et al., 2004; Callejo et al., 2006). In addition to their free diffusion, heparan sulfate proteoglycans (HSPGs) are thought to stabilize and recruit these ligands, with potential roles in promoting or, conversely, even limiting their diffusion (Yan and Lin, 2009). In addition, recent work has even suggested that filopodial-like extensions referred to as cytonemes may facilitate the spread of HH

ligands without actually releasing them from association with the signal-producing cell (Bischoff et al., 2013).

Signal transduction in the receiving cell (Fig. 1.5) is known to start with the binding or reception of HH ligands by the transmembrane receptor *Patched* (*Ptc1*). PTC1 normally represses pathway activity through the constitutive repression of *Smoothened* (*Smo*), yet upon binding of HH ligands, this repression is relieved. SMO is a transmembrane protein and member of the G protein-coupled receptor (GPCR) family, but has no identified ligand. Exactly how PTC1 regulates its activity is an open question, though the predominant view is that it controls the availability of a endogenous ligand, likely to be a sterol (Taipale et al., 2000; Corcoran and Scott, 2006; Dwyer et al., 2007; Nachtergaele et al., 2012), which then acts on SMO. Further, while this activation of Smo involves a conformational change (Zhao et al., 2007), it is unclear whether SMO utilizes G proteins, similar to other GPCRs (Ayers and Thérond, 2010). Ultimately, however, the relevant consequence of SMO activation is the resulting blockade of normal proteolytic processing required to generate repressor activity in the *Gli* family transcription factors.

In mice, both GLI2 and GLI3 contain zinc-finger DNA-binding domains, C-terminal activation domains, and N-terminal repression domains. It is the balance between activator and repressor forms of these factors that determines pathway activity. Interestingly, it is thought that the functions of GLI2 and GLI3 are somewhat divided between activation and repression, in that their mutant phenotypes resemble HH loss- and gain-of-function manipulations, respectively (Matise et al., 1998; Litingtung and Chiang, 2000). A third factor, GLI1, does not contain the N-terminal repressor domain, is not normally processed, and is therefore thought to play only a small role in potentiating the

response; accordingly, *Gli1* mutant mice are viable and phenotypically normal (Park et al., 2000). Instead, GLI1 transcription is often used as a read-out of pathway activity. In the absence of any signal, the GLI2 and GLI3 proteins are sequentially phosphorylated by a series of different kinases, which leads to ubiquitylation and partial degradation of the C-terminal activation domain. Thus, the remaining N-terminal portion serves as a transcriptional repressor, translocating to the nucleus and preventing the activation of downstream genes. Binding of HH and activation of SMO blocks this sequence of events from occurring, leaving GLI2 and GLI3 free to operate as transcriptional activators. Of note, this processing is also regulated by *Suppressor of Fused* (*Sufu*), which normally restrains or sequesters both GLI2 and GLI3 factors to promote their processing into repressor forms (Humke et al., 2010). The HH-mediated disruption of this interaction depends on KIF3A, a kinesin motor, and highlights a feature of the pathway unique to vertebrates: involvement of the primary cilium. Many different studies have now shown that the primary cilium is essential for vertebrate HH signal transduction (Goetz and Anderson, 2010). PTC1 localizes to the base of the primary cilium in the absence of bound ligand (Rohatgi et al., 2007), and is replaced in this location by SMO after pathway activation (Milenkovic et al., 2009; Wang et al., 2009). In addition, while transit of the GLI-SUFU complex through the cilium is essential for dissociation and subsequent pathway activation, it is also required for proteolytic processing and the maintenance of pathway repression (Liu et al., 2005; Endoh-Yamagami et al., 2009; Kim et al., 2009; Humke et al., 2010; He et al., 2014).

In conclusion, while certain aspects of the signal transduction process remain unclear, major proteins participating in and regulating the pathway are clear, and

manipulation of these factors has led to the proposition of several fairly well-defined roles in retinal development.

Roles as a mitogen and negative-feedback signal

SHH signaling is utilized iteratively throughout development in many different tissues, and one major theme of its function is use as a morphogen, responsible for patterning. In addition to roles in early eye development (including regionalization and patterning of the OC), SHH gradients are known to pattern the developing spinal cord (Dessaud et al., 2008) and limb (Bénazet and Zeller, 2009) of mammalian embryos. A discussion of these, and other context-specific roles, however, is beyond the scope of this introduction. Rather, it is necessary to focus for a moment on its known functions in later, neurogenic stages of eye development.

First, it is required to support adequate levels of proliferation during retinal neurogenesis and thus maintain a sizeable pool of progenitors, required for the generation of later-born cell types. As described above, work in *Drosophila* and zebrafish demonstrated that SHH promoted progression of the neurogenic wave in part by driving cell-cycle exit (Neumann and Nuesslein-Volhard, 2000; Shkumatava and Neumann, 2005). Those studies first documenting *Shh* expression in the murine retina, however, provided evidence to the contrary – that treatment of retinal cells in culture with recombinant SHH-N resulted in increased proliferation (Jensen and Wallace, 1997; Levine et al., 1997). Consistent with these results, genetic manipulations performed in mice resulted in increased proliferation (Moshiri and Reh, 2004; Wang et al., 2005). Using multiple pathway manipulations along with cell-cycle analysis, Locker and colleagues demonstrated that SHH sped up the cell cycle through reduction of G1 and G2

phases and promoted early cell-cycle exit (Locker et al., 2006). Thus, it was proposed that SHH mediated the production of fast cycling, transient amplifying progenitors (Locker et al., 2006; Agathocleous et al., 2007). Importantly, this is consistent with both early studies demonstrating a positive effect on proliferation as well as later studies demonstrating that such an effect was transient (Cwinn et al., 2011). This is related to experiments described in Chapter 4, and a further discussion of this topic can be found there.

Second, SHH is a well-documented negative feedback signal used to limit the generation of RGCs. Culture experiments in chick using conditioned media from differentially aged retinal cells first revealed that RGCs secrete a factor which limits their own generation (Waid and McLoon, 1998), and further studies revealed that increasing (via viral overexpression) and decreasing (via blocking antibody) pathway activity led to a decrease and increase, respectively, in RGC production (Zhang and Yang, 2001b). Similar to results describing proliferative effects (see above), these results contrasted somewhat with studies in both *Drosophila* and zebrafish that proposed SHH signaling functioned to positively promote both RGC production and, in turn, spread of the neurogenic wave (Nüsslein-Volhard and Wieschaus, 1980). Initial examination of SHH in the embryonic murine retina, however, provided support for its role as a negative feedback signal by showing that genetic inactivation led to the overproduction of RGCs (Wang et al., 2005). Multiple studies performed since, also mentioned above, have shown that both gain- and loss-of-function manipulations to the pathway have similarly expected effects, given a role in negative feedback (Sakagami et al., 2009; Cwinn et al., 2011). In

both Chapter 2 and Chapter 4, we detail a role for *Lhx2* in regulating RGC production and link this to a deficit in SHH signaling, further supporting these previous studies.

Notch signaling

Background and pathway components

Similar to the identification of *hedgehog*, *Notch* was first identified in *Drosophila*. Notch itself is a single-pass integral membrane protein, of which there are four in mice, encoded by the genes *Notch1-4*. In mammals, the post-translation processing and initial cleavage of Notch receptors results in the presentation of heterodimers at the cell surface (Blaumueller et al., 1997). Pathway activation (Fig. 1.6) requires binding of a ligand, of which several exist; in mice, these proteins are encoded by three separate *Delta-like* (*Dll1*, *3*, and *4*) and *Jagged* (*Jag1*, *2*) genes. Similar to the Notch receptors, these ligands are also single-pass integral membrane proteins. Thus, pathway activation depends on cell-cell contact. Binding of ligand and receptor drives dynamin-mediated endocytosis of both proteins in the signal-sending (Itoh et al., 2003; Overstreet et al., 2004; Wang and Struhl, 2004) and signal-receiving cells (Vaccari et al., 2008), respectively (Le Borgne et al., 2005; Fortini and Bilder, 2009). This results in physical deformation and a conformational change that exposes a previously unavailable cleavage site on the receptor (Parks et al., 2000; Gordon et al., 2007), initiating a series of proteolytic events in which Notch is cleaved at multiple locations by proteases of both the ADAM/TACE family (Brou et al., 2000) and γ-secretase complex (Fortini, 2002) to producing in sequence the Notch extracellular truncation (NEXT) (Mumm et al., 2000) and Notch intracellular domain (NICD). After release from the membrane, NICD translocates to the nucleus (Struhl and Adachi, 1998) and directly affects the transcription of target genes

through interaction with the CBF1/Su(H)/LAG-1 (CSL) transcription factors, as well as Mastermind and Mastermind-like coactivators (Petcherski and Kimble, 2000; Wu et al., 2000; Wilson and Kovall, 2006). In vertebrates, CBF1 is also known as RBPJ, and its role in developing RPCs is discussed in Chapter 3. The most well-characterized target genes are the aforementioned *Hes* and *Hes*-related bHLH transcription factors – notably *Hes1* and *Hes5*, among others.

The Notch pathway is relatively well characterized in mechanistic terms, yet in many contexts, including the retina, the exact functions have been difficult to discern due to both the complicated expression patterns and redundancy of various components. In spite of this, however, several overarching roles for Notch signaling in RPCs have emerged.

Progenitor cell maintenance and regulation of fate choice

Similar to Shh, Notch signaling is one of a select few pathways that serves a multitude of functions during development - far too numerous to cover here in adequate detail. However, general themes or functions of canonical Notch signaling are apparent, and include most prominently the regulation of fate decisions on a cellular level. Specifically, Notch signaling is heavily associated with the maintenance of progenitor populations and decision to differentiate in nervous system development (Artavanis-Tsakonas et al., 1995). This is a role that came out of many different studies in diverse model systems, and is centered on lateral inhibition. These traditional roles of Notch are arguably fulfilled in the retina, and thus we will introduce them in that context.

The activation of Notch signaling is consistently associated with RPC maintenance and proliferation in the vertebrate retina. Early misexpression studies

utilizing both ligands and receptors correlated pathway activity with an inhibition of differentiation in chick (Austin et al., 1995; Henrique et al., 1996; Ahmad et al., 1997), *Xenopus* (Dorsky et al., 1995; 1997), and rat (Bao and Cepko, 1997); shortly thereafter, the examination of both *Hes1* and *Hes5* mutant mice drew a similar conclusion (Tomita et al., 1996; Ohtsuka et al., 1999; Lee et al., 2005). Conditional inactivation allowed examination of many other factors in the developing mouse retina, and again, Notch activity was found to promote the maintenance and/or proliferation of RPCs (Jadhav et al., 2006; Yaron et al., 2006; Rocha et al., 2009; Zheng et al., 2009; Riesenberg et al., 2009b; Luo et al., 2012). Many of these later studies, in addition, had effects on the production of various individual cell types, highlighting potentially specific roles for the pathway components in the regulation of fate decisions (Riesenberg et al., 2009b).

While the conditional inactivation of different Notch pathway components in the retina often leads to the selective overproduction of one or more cell types, several different obstacles have prevented a clear picture of its function from emerging. These include incomplete definitions of cell-type specific expression patterns for individual components of the pathway, spatial and temporal variability in approach and analysis, as well as potential redundancy concerns. Perhaps the most well defined role exists for Notch1, which is thought to regulate photoreceptor differentiation. Conditional inactivation leads to an overproduction of photoreceptors (Jadhav et al., 2006; Yaron et al., 2006), and studies of late neurogenic RPCs have demonstrated that Notch activity is normally required to specify alternative fates (amacrine cells, bipolar cells, and Muller glia) at the expense of rod photoreceptors (Kechad et al., 2012; Mizeracka et al., 2013a; 2013b; Wang et al., 2014b). Carefully examining other components in a similar manner,

as well as clearly defining the fate decisions faced by RPCs (binary or otherwise) may yield a clearer picture of Notch function in the future.

Summary and goals

Early eye development is a complex process involving many different tissues – the growth, morphogenesis, and patterning of which are carefully coordinated on many different levels. As neurogenesis commences in the developing OC, an equally impressive coordination of proliferation and differentiation occurs such that RPCs are able to generate the correct number and proportion of every different cell type and subtype. Individual RPCs are multipotent, and though much is known about the factors required in these cells to generate the major retinal cell types, relatively little is known about 1) how stochastic or directed decisions are made to adopt different fates and 2) how the entire process is coordinated across a large population of cells, particularly in terms of competence regulation. Previously described function and expression data suggested that *Lhx2* may play a role in regulating this process in the retina, and the aim of this work was to thoroughly characterize the phenotype that results from loss of *Lhx2* function in RPCs. Along with identification of a mechanism, we also wished to develop a model for its function within this cellular population. To this end, several techniques were utilized, yet our studies were centered around conditional inactivation of the gene. The results detailed in the following chapters represent a significant contribution to our knowledge of several topics, including the function of *Lhx2* and the coordination of retinal neurogenesis. This work also provides insight into principles that may be utilized in many other biological contexts, and forms a foundation for future studies.

Figure 1.1 Schematic summary of early eye development. (A) Specification and bifurcation of the eye field are followed by expansion and evagination of the OV from the forebrain (arrows) (B) As the OV contacts the overlying surface ectoderm, lens development is initiated with formation of the lens placode, which subsequently begins to invaginate, in concert with the OV (arrows). (C) These morphological changes lead to formation of the lens vesicle and OC, respectively. During early OC stages, regionalization subdivides the neuroepithelial tissue into three distinct compartments: presumptive retina, RPE, and optic stalk. Abbreviations: SE, surface ectoderm; EOM, extraocular mesenchyme; OV, optic vesicle; LP, lens placode; LV, lens vesicle; pNR, presumptive neural retina; pRPE, presumptive retinal pigmented epithelium; pOS, presumptive optic stalk; D, dorsal; V, ventral. Approximates stages of development are listed at the bottom of each panel. Select gene expression patterns are show in italics.

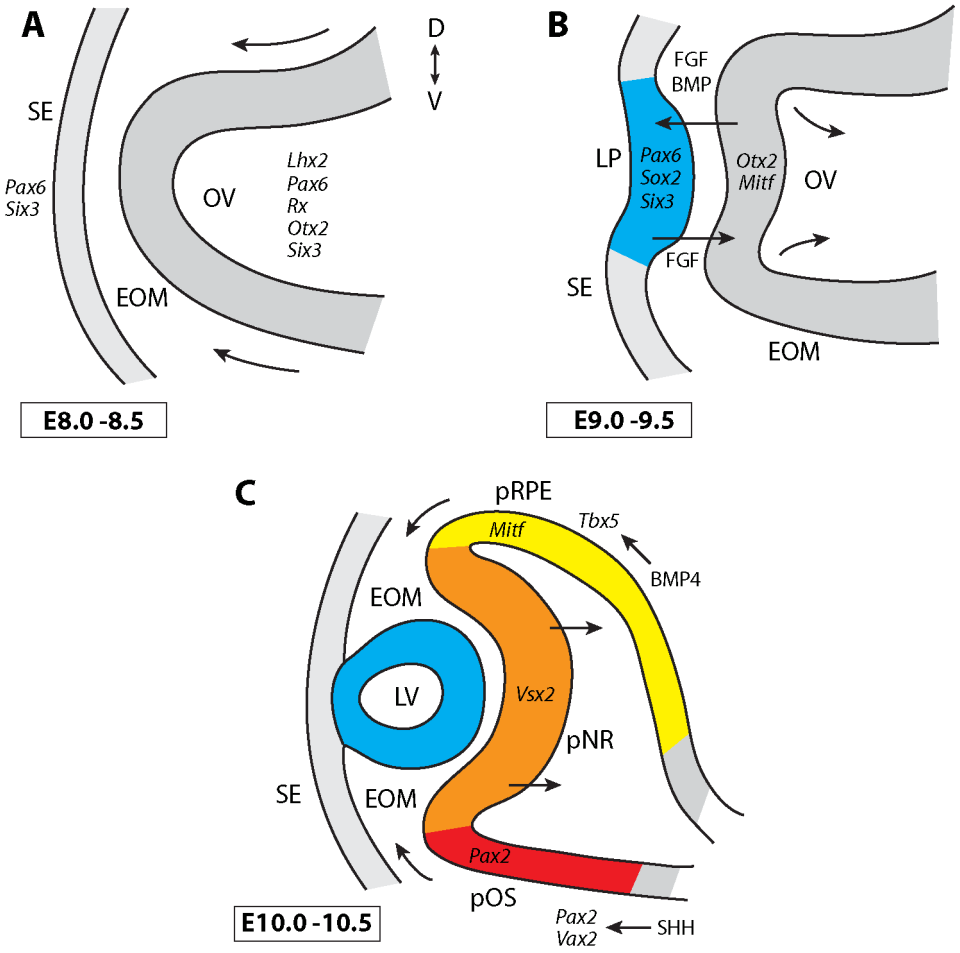

A
SE
Pax6
Six3
OV
Lhx2
Pax6
Rx
Otx2
Six3
EOM
D
V
E8.0 -8.5
B
FGF
BMP
LP
Pax6
Sox2
Six3
Otx2
Mitf
OV
FGF
SE
EOM
E9.0 -9.5
C
pRPE
Mitf
Tbx5
BMP4
EOM
LV
Vsx2
pNR
SE
EOM
Pax2
pOS
Pax2
Vax2
SHH
E10.0 -10.5

Figure 1.2 Initiation and propagation of neurogenesis in the retina. Following formation of the optic cup, neurogenesis initiates in the dorsal-central region of the murine retina, before spreading both ventrally and peripherally as a wavefront (arrows). The exact timing of initiation is likely dictated through a combination of both extracellular signals (Shh and Fgf from the optic stalk) and intrinsic factors (Sox2, Pax6, Vsx2, etc.). Ngn2 is required for the propagation of neurogenesis, and expression normally spreads ahead of both other proneural factors as well as markers of differentiation (green line). Atoh7 is another proneural bHLH heavily associated with RGC genesis, and its expression quickly follows that of Ngn2 (yellow line). In turn, both neuronal (Tubb3) and RGC-specific markers can be used to visualize the production of the first postmitotic neurons, accompanied by the production of Shh. Ascl1 expression initiates in the central retina at E12.5, marks lineage-restricted cells unable to generate RGCs, and is thought to drive a second wave of differentiation. Notch ligands Dll1 and Dll4 also spread in sequential waves, likely playing a role in the spread, and subsequent rate, of neurogenesis. Abbreviations: NBL, neuroblast layer; DCL, differentiated cell layer; D, dorsal; V, ventral.

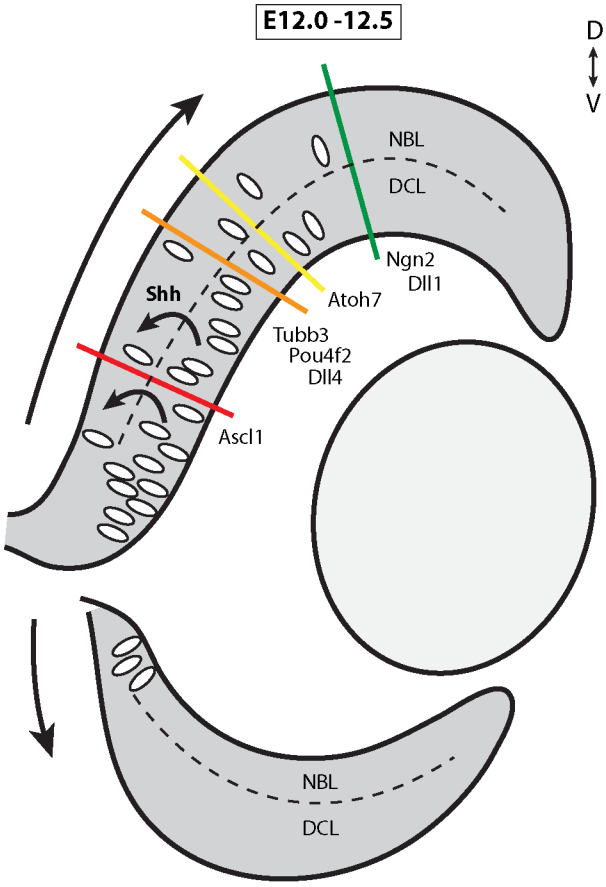

E12.0 -12.5
D
V
NBL
DCL
Ngn2
Dll1
Atoh7
Shh
Tubb3
Pou4f2
Dll4
Ascl1
NBL
DCL

Figure 1.3 Laminar organization of cell types in the mature retina. Light entering the eye traverses the thickness of the retina and is received by the sensory neurons of the eye, both cone and rod photoreceptors, located in the ONL. Photoreceptors pass this information directly to bipolar cells, through synapses located within the OPL. Bipolar cells, located within the INL, then pass the information to RGCs, through synapses located within the IPL. Horizontal and amacrine cells, both interneurons located within the INL, modulate this transmission of information. RGC axons compose the NFL, and exit the eye through the optic disc (not pictured). In addition to the neural cell types, Muller glia represent the major glial cell type of the retina, with cell bodies located in the INL and processes spanning the width of the tissue. Abbreviations: RPE, retinal pigmented epithelium; OS, outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer.

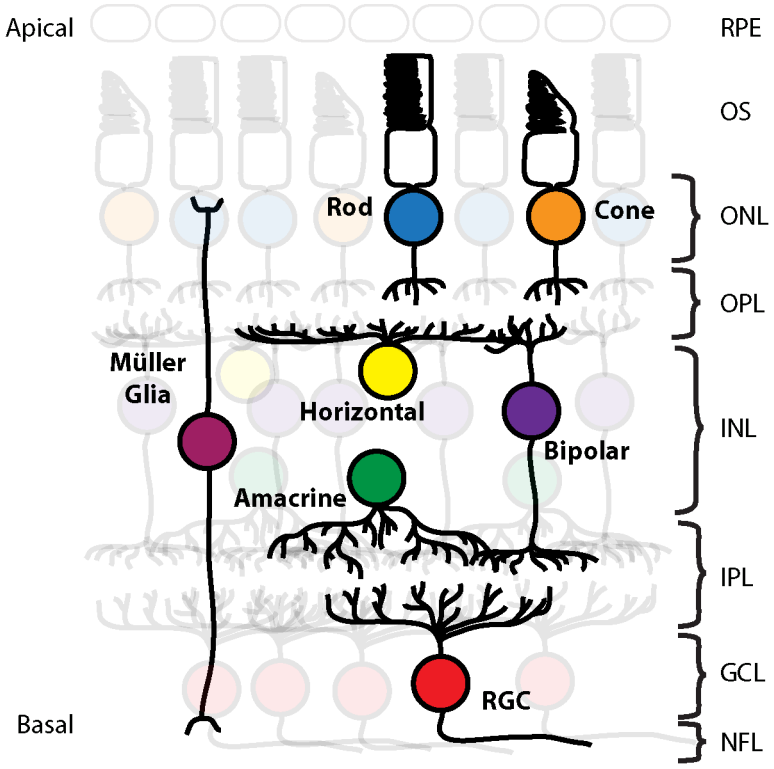

Apical
RPE
OS
Rod
Cone
ONL
OPL
Müller
Glia
Horizontal
Bipolar
INL
Amacrine
IPL
GCL
RGC
Basal
NFL

Figure 1.4 Summary of retinal histogenesis. (A) RPCs in the neural retina possess the potential (indicated by vertical, colored bars) to give rise to all major retinal cell types. As development proceeds, it is thought that each individual RPC proceeds irreversibly through a series of competence states; thus, at any given moment, RPCs leaving the cell-cycle (biased RPCs) are limited to the production of certain cell types. (B) The entire process takes approximately two weeks to complete, producing the various cell types of the mature retina during different windows, and in overlapping fashion. Abbreviations: RPC, retinal progenitor cell; RGC, retinal ganglion cell.

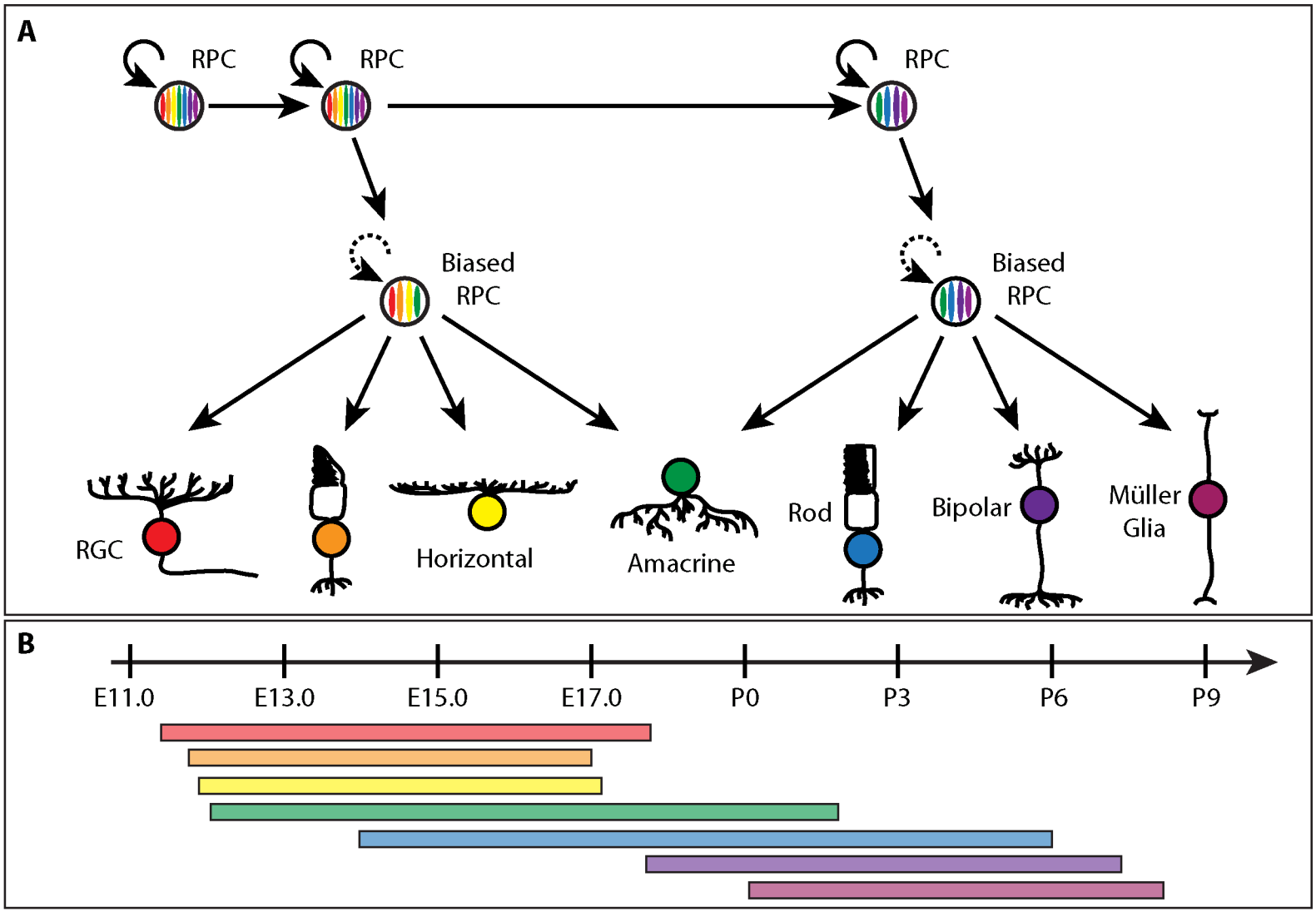

A
RPC
RPC
RPC
Biased
RPC
Biased
RPC
RGC
Horizontal
Amacrine
Rod
Bipolar
Müller
Glia
B
E11.0
E13.0
E15.0
E17.0
P0
P3
P6
P9

Figure 1.5 Summary of SHH signal transduction. SHH undergoes multiple post-translational modifications, thought to be important for the extracellular release and spread of the ligand, in various forms. In the signal receiving cell, transduction begins with binding of SHH to its canonical receptor PTC1; in certain cases, co-receptors (GAS1, CDON, and BOC) promote this interaction. This relieves constitutive inhibition of another transmembrane protein, SMO, which upon activation, disrupts the normal proteolytic processing of GLI2 and GLI3 transcription factors – a process normally promoted through interaction with Sufu. This allows them to enter the nucleus as transcriptional activators, activing various target genes – including *Gli1*, *Ptc1*, and *Hhip1*.

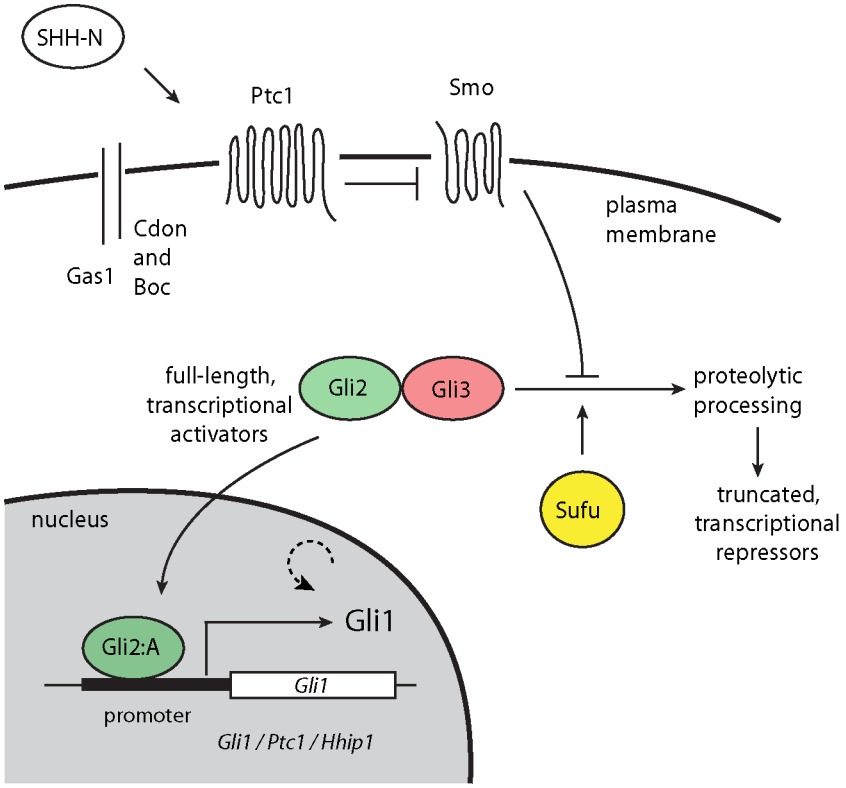
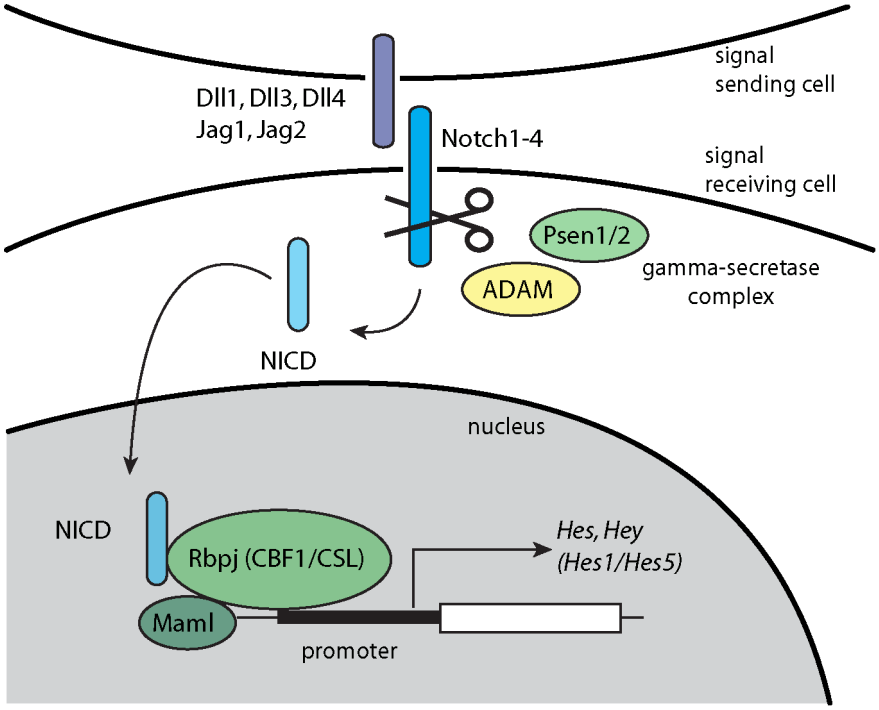

SHH-N
Ptc1
Smo
plasma
membrane
Cdon
and
Boc
Gas1
full-length,
transcriptional
activators
Gli2
Gli3
proteolytic
processing
truncated,
transcriptional
repressors
Sufu
nucleus
Gli1
Gli2:A
Gli1
promoter
Gli1 / Ptc1 / Hhip1

Figure 1.6 Summary of Notch signal transduction. Both ligand (*Dll1*, *3*, *4* and *Jag1*, *2*) and receptor components (*Notch1-4*) in the Notch signaling pathway are single-pass transmembrane proteins, and thus, signaling activity requires cell-cell contacts. Both ligand and receptor are internalized upon binding, generating a physical deformation that exposes a cleavage site in the Notch receptor, allowing for cleavage in multiple locations and ultimately releasing the intracellular component (NICD). This portion of the receptor translocates to the nucleus and affects transcription of target genes (*Hes*/*Hey*) through interaction with the canonical effector RBPJ as well as co-activators.


signal
sending cell
Dll1, Dll3, Dll4
Jag1, Jag2
Notch1-4
signal
receiving cell
Psen1/2
gamma-secretase
complex
ADAM
NICD
nucleus
NICD
Rbpj (CBF1/CSL)
Hes, Hey
(Hes1/Hes5)
Maml
promoter

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CHAPTER 2

LHX2 BALANCES PROGENITOR MAINTENANCE WITH NEUROGENIC OUTPUT AND PROMOTES COMPETENCE STATE PROGRESSION IN THE DEVELOPING RETINA

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Figure 1, Figure 2, Figure 3 (A-L and S-Z), and Figure 4, along with all of the associated experiments, were performed by Sanghee Yun, who is a co-first author on the published article cited above. In addition, these figures and associated experiments have previously been published as part of her doctoral thesis:

Yun S (2009) The roles of the LIM homeobox gene Lhx2 in early eye development and retinal histogenesis.

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Development/Plasticity/Repair

Lhx2 Balances Progenitor Maintenance with Neurogenic Output and Promotes Competence State Progression in the Developing Retina

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The LIM-Homeodomain transcription factor *Lhx2* is an essential organizer of early eye development and is subsequently expressed in retinal progenitor cells (RPCs). To determine its requirement in RPCs, we performed a temporal series of conditional inactivations in mice with the early RPC driver *Pax6 α-Cre* and the tamoxifen-inducible *Hes1*^{CreERT2} driver. Deletion of *Lhx2* caused a significant reduction of the progenitor population and a corresponding increase in neurogenesis. Precursor fate choice correlated with the time of inactivation; early and late inactivation led to the overproduction of retinal ganglion cells (RGCs) and rod photoreceptors, respectively. In each case, however, the overproduction was selective, occurring at the expense of other cell types and indicating a role for *Lhx2* in generating cell type diversity. RPCs that persisted in the absence of *Lhx2* continued to generate RGC precursors beyond their normal production window, suggesting that *Lhx2* facilitates a transition in competence state. These results identify *Lhx2* as a key regulator of RPC properties that contribute to the ordered production of multiple cell types during retinal tissue formation.

Introduction

The ordered production of multiple cell types from a single pool of progenitor cells is a common strategy used in the developing vertebrate nervous system. However, the regulation of multipotent stem and progenitor cell populations is complex, requiring robust control and coordination to ensure reproducible outcomes. The retina has long served as a model for the study of ordered cell production, as the seven major cell types (retinal ganglion cells (RGC), cone photoreceptor, horizontal, amacrine, rod photoreceptor, bipolar, and Müller glia) arise in a successive yet overlapping sequence from a common pool of multipotent retinal progenitor cells (RPCs; Turner and Cepko, 1987; Holt et al., 1988; Turner et al., 1990; Rapaport et al., 2004; Wong and Rapaport, 2009). Still unresolved, however, is how RPCs control their developmental potential to generate precursors of each cell type at the correct time and in their correct proportions.

Two dominant models have emerged: one is based on competence changes and proposes that RPCs are equipotent, but transition irreversibly through a series of intrinsically defined states during which they are capable of generating specific cell types (Cepko et al., 1996). The other is based on progressive lineage restriction, arguing for the existence of RPC cohorts with unequal fate potential, identifiable on the basis of selective gene expression (Li et al., 2004; Vitorino et al., 2009; Brzezinski et al., 2011; Hafler et al., 2012). Both models portray RPCs as predictable, yet direct observations show their behavior to be stochastic (i.e., decisions regarding division mode and fate choice cannot be predicted on the basis of history or lineage) (Gomes et al., 2011; He et al., 2012). RPCs do not behave randomly, however, as all outcomes are not chosen with equal probability. As a result, mechanisms must exist to set and modulate the relative probability of competing outcomes.

Lhx2 is used in many different tissues, with context-dependent functions including the regulation of regional identity and the maintenance of stem cell character (Rhee et al., 2006; Dahl et al., 2008; Mangale et al., 2008). It is expressed early in the developing eye and required for regionalization, patterning, and lens formation (Yun et al., 2009); consequently, eye development arrests in *Lhx2*$^{-/-}$ mice (Porter et al., 1997) and the retinal domain is never specified. *Lhx2* is also expressed in RPCs during histogenesis, and given that it regulates fate decisions elsewhere, we predicted that *Lhx2* would contribute to regulation of neurogenesis in the retina. Using *Pax6 α-Cre* and the tamoxifen-inducible *Hes1*^{CreERT2}, we performed a temporal series of

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conditional inactivations and uncovered requirements for *Lhx2* in regulating RPC maintenance, output, and competence.

Materials and Methods

Animals. The *Lhx2* conditional allele was generated by Mangale et al., (2008), *Pax6 α-Cre* was generated by Marquardt et al. (2001), and the *Hes1*^{CreERT2} knock-in allele was generated by Kopinke et al. (2011). *R26R* (Soriano, 1999) and *Ai14 tdTomato* (Madisen et al., 2010) reporter mice were obtained from The Jackson Laboratory. *Hes1*$^{CreERT2/+}$;*Lhx2*$^{f/+}$ mice were used as a control for *Hes1*$^{CreERT2/+}$;*Lhx*$^{f/-}$. *α-Cre;Lhx2*$^{f/+}$ mice were used as controls for *α-Cre;Lhx2*$^{f/-}$ mice. The *R26R* and *Ai14 tdTomato* alleles were used as heterozygotes. Embryonic age determinations were based on plug date. Tissues were collected from mice of either sex. All animal use and care was conducted in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee and set forth in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals. Efforts were made to minimize discomfort to animals and when possible, the number of animals needed per analysis was kept to a minimum.

Administration of tamoxifen and 5-ethynyl-2′-deoxyuridine. Tamoxifen (Sigma) was dissolved in corn oil (Sigma) at a concentration of 10 mg/ml. For widespread activation of Cre recombinase in *Hes1*^{CreERT2} mice, 0.1 mg TM/g body weight was administered into pregnant dams by oral gavage at various embryonic stages with 22 G 1.5 inch feeding needle. The 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) was dissolved in filtered PBS at a concentration of 10 mM and administered via intraperitoneal injection. Pregnant dams were given a single injection of 30 μg/g body weight, 24 h before sacrifice.

Immunohistochemistry. Embryo heads or eyes were dissected in HBSS or PBS and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature or 2 h on ice. Following fixation, tissue was washed twice with PBS, put through a gradient of sucrose solutions, embedded in OCT (Sakura Finetek), and stored at −80°C. Frozen tissues were sectioned on a cryostat at a thickness of 12 μm. Primary antibodies used were as follows: anti-LHX2 (Edwin Monuki, University of California, Irvine; 1:50), anti-LHX2 (Santa Cruz Biotechnology; 1:1000), anti-Calretinin (Millipore Bioscience Research Reagents; 1:1000), anti-P27 (BD Bioscience; 1:100), anti-CCND1 (Lab Vision; 1:400), anti-PCNA (DAKO; 1:500), anti-BRN3 (Santa Cruz Biotechnology; 1:50), anti-SOX2 (Abcam; 1:400), anti-AQP4 (Santa Cruz Biotechnology; 1:300), anti-RXRγ (Santa Cruz Biotechnology; 1:200), anti-NR2E3 (Anand Swaroop, National Eye Institute; 1:100), anti-BHLHB5 (Santa Cruz Biotechnology; 1:1000), anti-GABA (Sigma; 1:1000), anti-VSX2 (Exα Biologicals; 1:300), anti-VSX1 (Clark et al., 2008; 1:500), anti-SOX9 (Millipore Bioscience Research Reagents; 1:400), anti-CRALBP (John Saari, University of Washington; 1:1000), anti-GFAP (Lipshaw; 1:1000), anti-βIII-Tubulin (Covance; 1:1000), anti-l^{y}TF1A (Helena Edlund, Umea University; 1:800), anti-PTF1A (Jane Johnson, University of Texas Southwestern; 1:8000), and anti-OTX2 (Millipore Bioscience Research Reagents; 1:15,000). Primary antibodies were followed with species-specific secondary antibodies conjugated to either Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 (Invitrogen), or donkey anti-goat IgG conjugated with tetramethylrhodamine isothiocyanate (Jackson ImmunoResearch). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Fluka). Panels showing fluorescence-based protein detection are single-scan confocal images from a Fluoview 1000 confocal microscope (Olympus).

In situ *hybridization.* Embryo heads were dissected in HBSS and fixed overnight at 4°C in 4% PFA in PBS, washed twice with PBS, put through a gradient of sucrose solutions, embedded in OCT, and stored at −80°C. Frozen tissues were sectioned on a cryostat at 12 μm in the coronal plane, and *in situ* hybridization was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). Probes used in this study were digoxigenin-labeled antisense probes against *Hes1*, *Hes5*, and *Atoh7*.

Quantification and statistical analyses. Retinal thickness was calculated manually in Photoshop as the average of three separate and equally spaced measures (individual lines oriented perpendicular to the apical membrane and spanning the entire width of the retina) of each image. For lineage tracing experiments, different cell types were identified based on the expression of precursor-specific markers. The percentage of *Lhx2*-inactivated RPCs that assumed any particular fate was calculated as the number of marker-positive, reporter-positive cells over total reporter-positive cells. As E12.5 inactivation produced embryos with an asymmetric phenotype along the dorsoventral axis (see Fig. 6), only the more affected sides of each retina, as determined by hierarchical clustering, were included in our quantification of neurogenic output in Figure 5. Birth-dating experiments were quantified using a similar method: the absolute number of Pou4f+EdU+ cells was counted on the more affected sides of each experimental retina and expressed as a count per unit area. Area was calculated in Photoshop. Hierarchical clustering in Figure 6*F* was performed using Ward's method, and distance joined to form each iterative cluster is depicted in both the scale of the dendrogram as well as the scree plot underneath. For determining significance in all comparisons we used an α level of 0.05 and a two-sided Aspin–Welch–Satterthwaite–Student's t test, assuming unequal variance. JMP 10.0 software was used for all calculations as well as generation of the dendrogram and scree plot. All data are shown as the mean ± SE.

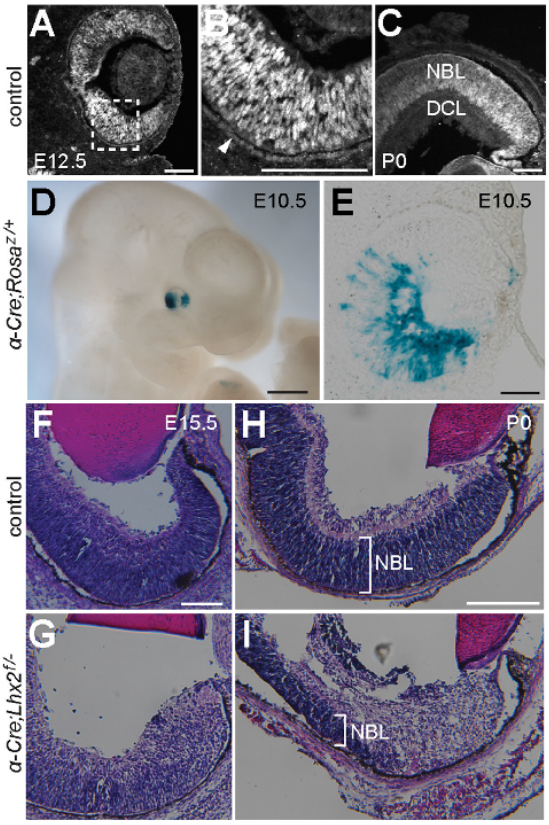

Figure 1. Lhx2 is expressed in RPCs during embryonic stages and inactivation with *α-Cre* results in profound mispatterning. ***A***, ***B***, Control sections stained for Lhx2 show expression in most if not all RPCs, as well as the retinal pigment epithelium (arrowhead; ***B*** shows boxed area in ***A***). ***C***, At later ages, expression is limited to the NBL and absent from the DCL. ***D***, ***E***, *α-Cre* drives recombination in the ventral and peripheral retina, evident by crossing *α-Cre* with *R26R* and staining with X-gal. ***F–I***, Histologic sections show that *α-Cre;Lhx2*$^{f/-}$ eyes are disorganized compared with controls. Scale bars: ***A–C***, ***E–I***, 100 μm; ***D***, 1 mm.

Results

Expression of *Lhx2* in the embryonic and postnatal retina

We found that during embryonic development Lhx2 was expressed in most if not all RPCs within the neuroblast layer (NBL) of the retina, as well as in the retinal pigment epithelium (Fig.

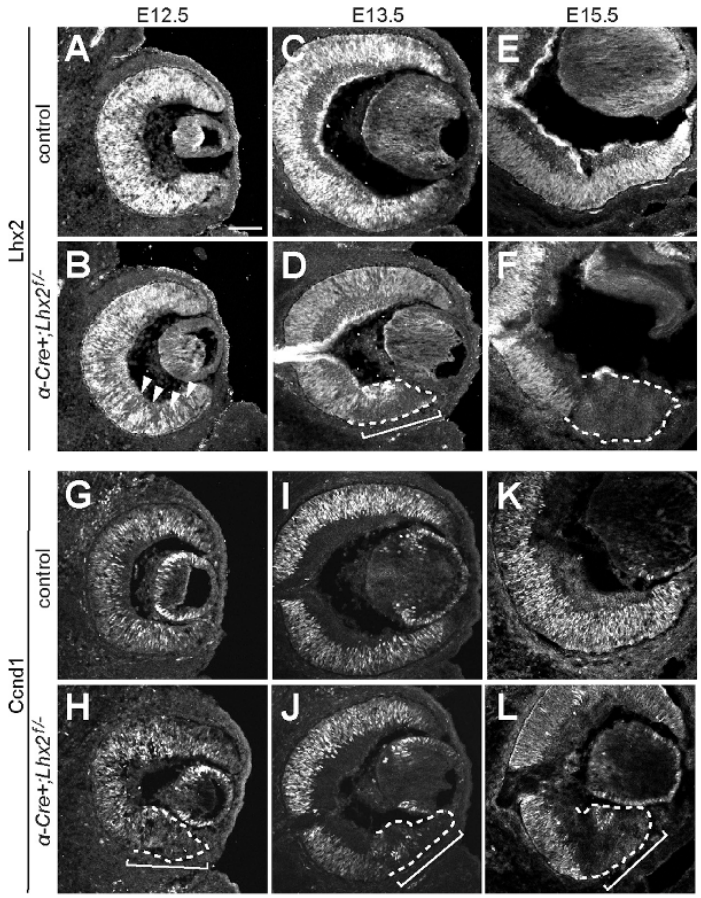

Figure 2. Loss of *Lhx2* results in the significant depletion of RPCs. ***A–L***, Sections from control and α-*Cre;Lhx2*$^{f/-}$ eyes stained with antibodies against Lhx2 (***A–F***) and Ccnd1 (***G–L***) show that loss of Lhx2 is first evident in patches (***B***, arrowheads) before becoming more obvious at later ages (dashed lines and brackets in ***D***, ***F***). Ccnd1 expression is lost in a similar manner (dashed lines and brackets in ***H***, ***J***, ***L***). Scale bar, 100 μm.

1 *A*, *B*; arrowhead). Expression was lost upon differentiation, as Lhx2 was notably absent from the differentiated cell layer (DCL; Fig. 1*C*). Postnatally, expression was maintained in Müller glia and a subset of amacrine cells, indicated by colocalization with p27^{Kip1} and Calretinin, respectively (data not shown). Both embryonic and postnatal expression patterns described here correspond with previous findings in mice (Tétreault et al., 2009; de Melo et al., 2012) as well as other species (Seth et al., 2006; Viczian et al., 2006), suggesting a conserved role for *Lhx2*.

Loss of *Lhx2* depleted the RPC pool and increased neurogenesis

To bypass the essential roles of *Lhx2* during early eye development we conditionally inactivated a floxed allele of *Lhx2* (Mangale et al., 2008) using the *Pax6* α-*Cre* retinal driver (Marquardt et al., 2001), in which a retina-specific enhancer element (α) from the *Pax6* locus drives expression of Cre recombinase in the ventral peripheral retina from E10.5 onward (Kammandel et al., 1999; Marquardt et al., 2001; Fig. 1 *D*,*E*). This onset of expression was appropriate for the study of neurogenic stages, as it occurs 1 d after the developmental arrest in *Lhx2*$^{-/-}$ mice and also coincides with the approximate beginning of neurogenesis. Initial inspection and subsequent H&E staining of α-*Cre;Lhx2*$^{f/-}$ eyes revealed aberrant morphology as early as E15.5 (Fig. 1*F*,*G*), with the laminar architecture of the retina disrupted and the NBL reduced in thickness by P0 (Fig. 1*H*,*I*). Loss of Lhx2 in α-*Cre;Lhx2*$^{f/-}$ eyes was observed in the ventral retina as early as E12.5 (Fig. 2*A*,*B*, arrowheads), and became more widespread by E13.5 and E15.5 (Fig. 2*C–F*, dashed lines and brackets). To assess the status of proliferative RPCs, we examined expression of *Cyclin D1* (Ccnd1), present in the majority of RPCs during neurogenesis (Barton and Levine, 2008; Das et al., 2009, 2012) (Fig. 2*G*,*I*,*K*). Ccnd1 expression was lost as early as E12.5 (Fig. 2*H*, dashed line and bracket) and almost completely absent by E13.5 (Fig. 2*J*, dashed line and bracket) and E15.5 (Fig. 2*L*, dashed line and bracket). These observations suggest that *Lhx2* is required for the maintenance and/or proliferation of RPCs during neurogenesis.

To determine whether RPCs were undergoing apoptosis, we examined the relative number of Tunel+ cells and found an increase at P0 but not E13.5 (data not shown). This suggested that *Lhx2*-inactivated RPCs were not immediately lost to cell death, but rather may have entered a quiescent state or undergone premature differentiation. As indicated by the enhanced expression of class-III β-Tubulin (Tubb3), a marker of postmitotic differentiating neurons, *Lhx2*-inactivated RPCs appeared to precociously differentiate (Fig. 3*A–F*). Within the ventral retina, Tubb3+ cells were observed ahead of the normal wave of neurogenesis at E12.5 (Fig. 3*B*, arrowheads) and present in increased numbers at later time points (Fig. 3*D*,*F*), consistent with the loss of Lhx2 in that region (Fig. 2*B*,*D*,*F*). Together, the depletion of RPCs and reciprocal increase in neurogenesis suggested that *Lhx2* normally plays a role in promoting the maintenance of RPCs.

Lhx2 inactivation at E10.5 resulted in selective overproduction of RGCs

To identify the fates of *Lhx2*-inactivated RPCs, we examined markers associated with precursor fate selection. An antibody against the RGC-specific markers *Pou4f1*, *2*, and *3* (hereafter referred to as *Pou4f*) showed a pattern similar to that of Tubb3 in α-*Cre;Lhx2*$^{f/-}$ eyes: Pou4f+ cells were observed ahead of the normal wave of neurogenesis at E12.5, again confined to the inactivated region of the ventral retina (Fig. 3*H*, arrowheads). At later time points (E15.5, P0) Pou4f+ cells were more abundant (Fig. 3*I–L*) and widely distributed while the NBL was much thinner (Fig. 3*K*,*L*, brackets), supporting the notion that ectopic RGCs were produced at the expense of the RPC pool. To determine whether *Lhx2* was acting upstream of genes important for RGC specification, we examined expression of *Atoh7* (formerly *Math5*) and *Hes1*. *Atoh7* is a proneural bHLH transcription factor, necessary yet not suf-

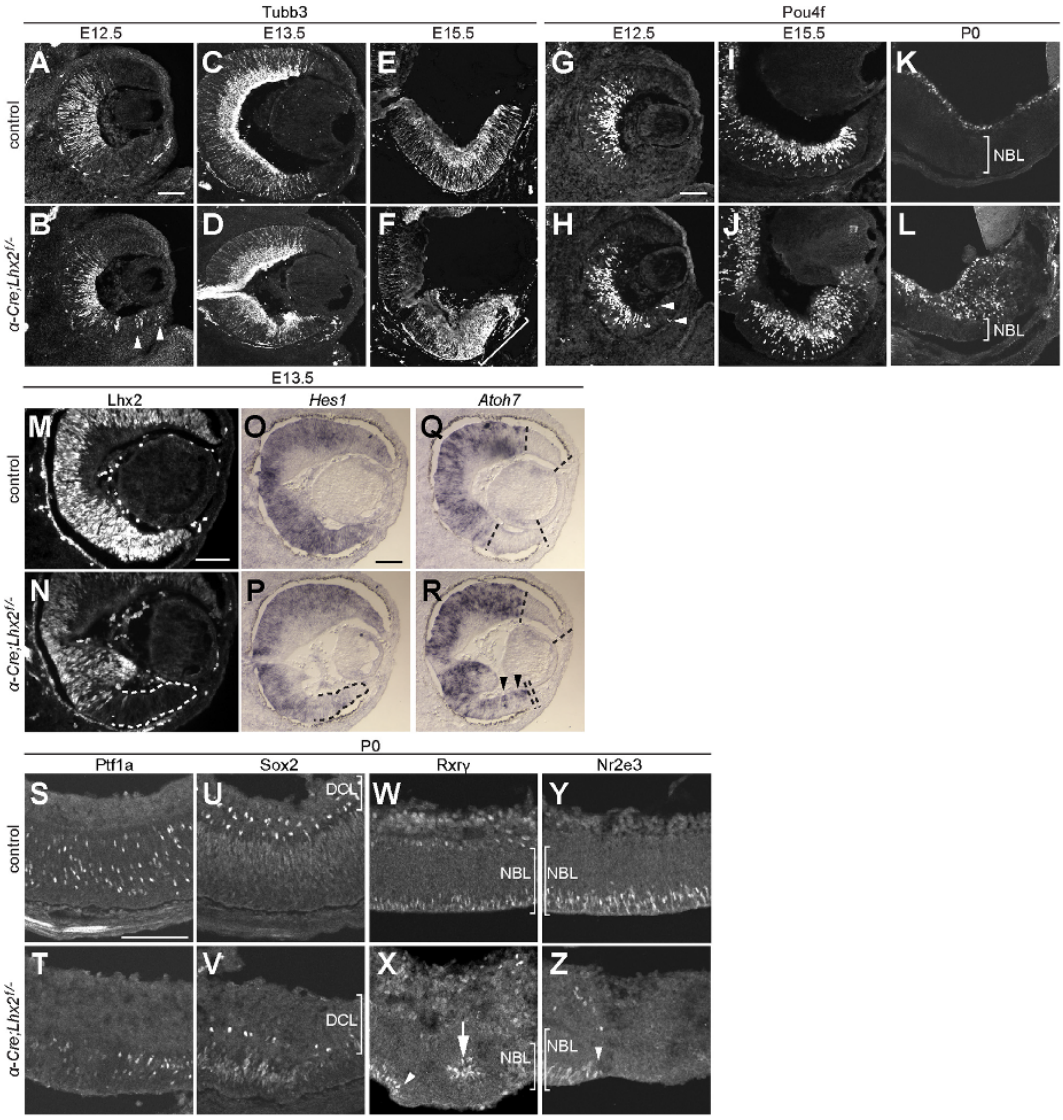

Figure 3. Inactivation of *Lhx2* with α-*Cre* results in the selective overproduction of RGCs. ***A–L***, Sections from control and α-*Cre;Lhx2*$^{f/-}$ eyes stained with antibodies against Tubb3 (differentiated cells, ***A–F***) and Pou4f (RGC precursors, ***G–L***) show that premature (arrowheads in ***B***, ***H***) and increased neurogenesis in the ventral retina is accounted for by RGC precursors and occurs at the expense of RPCs (brackets in ***K***, ***L***). ***M–R***, Immunostaining and *in situ* hybridization demonstrate that the loss of Lhx2 (dashed line in ***N***) is accompanied by loss of *Hes1* (dashed line in ***P***) and precocious *Atoh7* expression (arrowheads, dashed lines in ***Q***, ***R***). ***S–Z***, Immunostaining for Ptf1a (horizontal and amacrine cell precursors, ***S***, ***T***), Sox2 (amacrine cell precursors, ***U***, ***V***), Rxrγ (cone precursors, ***W***, ***X***), and Nr2e3 (rod precursors, ***Y***, ***Z***) demonstrates that RGC overproduction coincides with the underproduction of other early born and later born precursor types. ***X***, ***Z***, Arrowheads mark the presumptive boundary of recombination; arrow highlights cone precursors organized into a rosette. Scale bars: 100 μm.

ficient for RGC formation (Brown et al., 1998, 2001). *Hes1* is another bHLH transcription factor that acts upstream of *Atoh7* to inhibit differentiation, and *Hes1* mutants show precocious activation of *Atoh7* and increased RGC production (Lee et al., 2005; Riesenberg et al., 2009). We examined α-*Cre;Lhx2*$^{f/-}$ retinas at E13.5, when normal *Atoh7* expression has not yet reached the periphery, giving us the opportunity to observe precocious activation. α-*Cre;Lhx2*$^{f/-}$ eyes showed the expected changes: loss of Lhx2 (Fig. 3*M*,*N*, dashed line) and *Hes1* (Fig. 3*O*,*P*, dashed line) along with precocious *Atoh7* expression (Fig. 3*Q*,*R*, arrowheads). These results demonstrate that *Lhx2* acts at or above the level of *Hes1*, itself an important target of several signaling pathways known to promote the maintenance of progenitor and/or stem cell populations.

In contrast to the change in Pou4f+ RGC precursors, precursors of other cell types were noticeably decreased in the ventral retina at P0. This included Ptf1a+ horizontal and amacrine cell precursors (Fig. 3*S*,*T*), the Sox2+ subset of amacrine cells in the DCL (Fig. 3*U*,*V*), Rxrγ+ cone photoreceptors in the NBL (Fig. 3*W*,*X*), and Nr2e3+ rod photoreceptors in the NBL (Fig. 3*Y*,*Z*). While underproduction of the later born rod

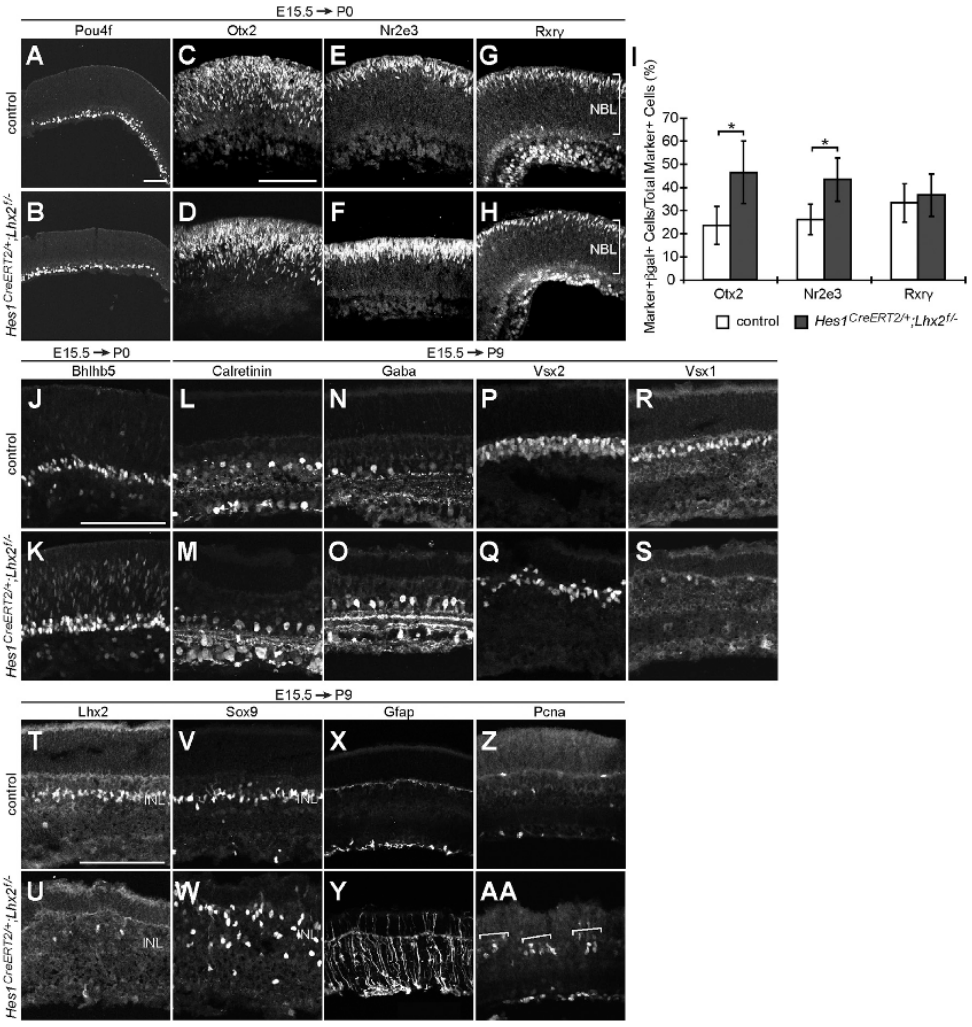

Figure 4. Inactivation of *Lhx2* with $Hes1^{CreERT2}$ at E15.5 results in the overproduction of rods. ***A–H***, Sections from control and $Hes1^{CreERT2/+}$;$Lhx2^{f/-}$ eyes stained with antibodies against Pou4f (RGC precursors, ***A***, ***B***), Otx2 (photoreceptor precursors, ***C***, ***D***), Nr2e3 (rod precursors, ***E***, ***F***), and Rxrγ (cone precursors, ***G***, ***H***). ***I***, The number of RGC precursors is unchanged, while the number of photoreceptor precursors in the NBL is increased due to the selective overproduction of rods (*$p = 0.0007$ and *$p = 0.0004$, respectively; $n = 3$ mice for each genotype; $n > 200$ marker+ cells for each genotype). ***J–O***, Immunostaining for Bhlhb5 (***J***, ***K***), Calretinin (***L***, ***M***), and Gaba (***N***, ***O***) show that amacrine cell precursors are slightly increased. ***P–S***, Immunostaining for Vsx2 (***P***, ***Q***) and Vsx1 (***R***, ***S***) show that bipolar cells are decreased. ***T–W***, At P9, Lhx2, predominantly expressed in Müller glia, is largely absent in the $Hes1^{CreERT2/+}$;$Lhx2^{f/-}$ retina (***T***, ***U***). Müller glia are still present, but disorganized, as indicated by Sox9 (***V***, ***W***). ***X–AA***, Immunostaining for Gfap (***X***, ***Y***) and Pcna (***Z***, ***AA***) show that the *Lhx2*-inactivated Müller glia are reactive and proliferative. Time points above each part indicate the time of inactivation and analysis, respectively. Scale bars:100 μm. INL, inner nuclear layer.

photoreceptor precursors could arise indirectly from RPC depletion, the underproduction of early born cell types demonstrates a selective effect on fate choice, as their production windows normally overlap with that of RGCs. These observations suggest that *Lhx2* has an important role in not only maintaining the RPC population, but also in promoting the generation of several early born precursor types by normally limiting RGC precursor production.

Lhx2 inactivation at E15.5 resulted in overproduction of later born cell types, without affecting RGCs

To determine whether *Lhx2* regulates the production of diverse fates during later stages of retinal neurogenesis, we used $Hes1^{CreERT2}$, a knock-in allele in which tamoxifen-dependent CreERT2 is expressed under the regulatory control of the *Hes1* gene locus (Yun et al., 2009; Kopinke et al., 2011). Confirming the results obtained with α-*Cre*, administration of tamoxifen at E10.5 resulted in RPC depletion and RGC overproduction; however, the phenotype was observed throughout the retina, consistent with the broad domain of recombinase activity (data not shown). We next shifted the time of inactivation to E15.5, when the peak of RGC production has passed and rod and amacrine cell precursors are the predominant cells produced. In examining $Hes1^{CreERT2/+}$;$Lhx2^{f/-}$ eyes at P0, the profile of Pou4f+ RGCs was not altered (Fig. 4*A*,*B*). Instead, the number of Otx2+ photoreceptor precursors had increased (Fig. 4*C*,*D*). This was due pri-

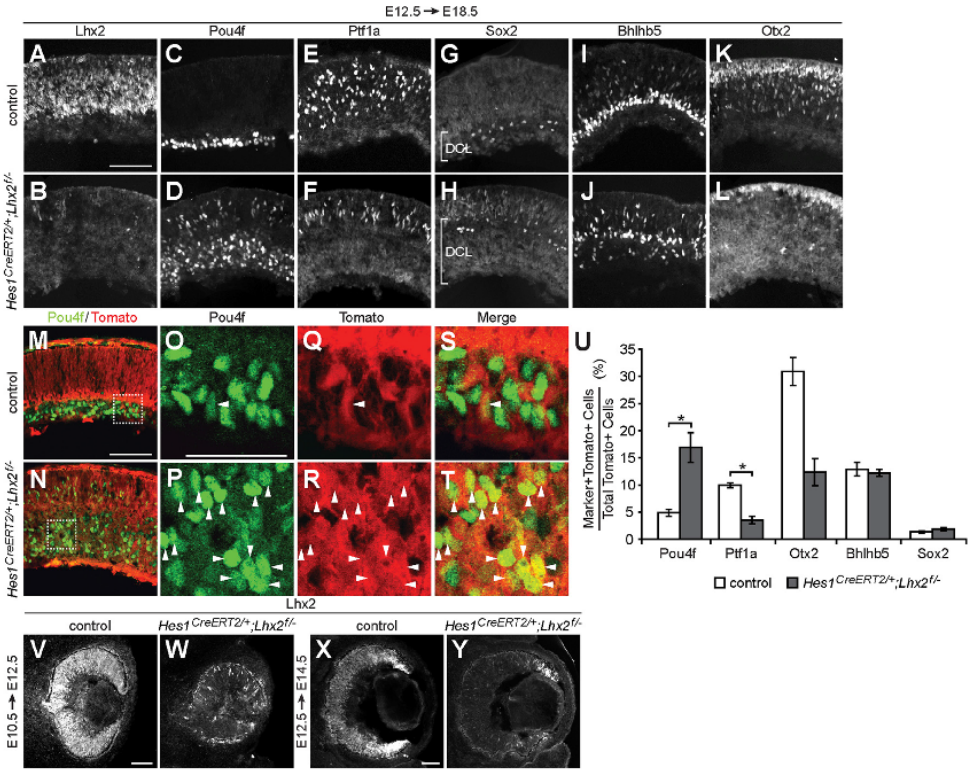

Figure 5. Inactivation of *Lhx2* at E12.5 does not result in overproduction of other early born cell types. ***A–L***, Sections from control and $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes stained with antibodies against Lhx2, Pou4f (RGC precursors, ***C***, ***D***), Ptf1a (horizontal and amacrine cell precursors, ***E***, ***F***), Sox2 (amacrine cell precursors, ***G***, ***H***), Bhlhb5 (amacrine cell precursors, ***I***, ***J***), and Otx2 (photoreceptor precursors, ***K***, ***L***) show that, similar to inactivation at E10.5, RGC precursors are selectively overproduced while other early born cell types are unchanged or decreased in number. ***M–T***, The progeny of *Lhx2*-inactivated RPCs are more likely to adopt the RGC fate and subsequently express the precursor marker Pou4f (arrowheads, ***O–T***). Tomato expression marks the recombined population in both control and $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes (***O***, ***Q***, and ***S*** show the boxed area in ***M***; ***P***, ***R***, and ***T*** show the boxed area in ***N***). ***U***, The percentage of the recombined population expressing Pou4f+ is significantly increased while the percentages expressing Ptf1a+ and Otx2+ are decreased (*$p = 0.0429$, *$p = 0.0021$, and $p = 0.066$, respectively; $n = 3$ mice for each genotype; $n > 1000$ Tomato+ cells for each experiment). ***V–Y***, Lhx2 inactivation proceeds with similar kinetics whether initiated at E10.5 (***V***, ***W***) or E12.5 (***X***, ***Y***). In each case, only a few Lhx2+ cells remain after 2 d. Error bars indicate SEM. Scale bars: 100 μm.

marily to enhanced rod precursor production, as revealed by the increased expression of Nr2e3 (Fig. 4*E*,*F*), and minimal effect, if any, on cone precursor production, as revealed by Rxrγ expression (Fig. 4*G*,*H*). We confirmed these observations by using the *R26R* recombination reporter (Soriano, 1999) to mark the progeny of *Lhx2*-inactivated RPCs. Indeed, inactivated RPCs contributed a significantly higher percentage of cells to the Otx2+ and Nr2e3+ precursor populations in $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes (Fig. 4*I*). The relative abundance of cells expressing amacrine cell markers at both P0 and P9 was generally unchanged or modestly increased (Fig. 4*J–O*). Bipolar cells, however, were severely reduced in number as indicated by Vsx1 and Vsx2 (formerly Chx10; Fig. 4*P–S*). We expected a similar reduction in the number of Müller glia, yet while expression of Lhx2 in the inner nuclear layer was expectedly absent due to inactivation (Fig. 4*T*,*U*), their numbers remained relatively unchanged at P9 as assessed with Sox9 (Fig. 4*V*,*W*). Instead, Müller glia were proliferative and reactive as indicated by Pcna (Fig. 4*Z*,*AA*), Gfap (Fig. 4*X*,*Y*), and the displacement of Sox9+ cells (Fig. 4*V*,*W*). These effects on Müller glia are similar to the recently reported inactivation of *Lhx2* in adult Müller glia, with the exception that evidence of proliferation was not observed (de Melo et al., 2012). This difference could be due to a nonautonomous response by the Müller glia to the alterations in retinal histogenesis caused by *Lhx2* inactivation in E15.5 RPCs. Regarding neural cell types, however, inactivation at E10.5 and E15.5 together demonstrate that *Lhx2* regulates diversity and ensures that the major cell types of the retina are generated in their correct proportions.

RGC overproduction predominates after *Lhx2* inactivation at E12.5

To test further the temporal dependence of cell-type production in response to *Lhx2* inactivation, we shifted the tamoxifen treatment time to E12.5 and determined the neurogenic output at E18.5, a time chosen to allow adequate recombination and precursor generation, yet also to avoid the complicating increase in cell death observed at P0 in α-$Cre;Lhx2^{f/-}$ eyes. Since production of horizontal, cone, and amacrine cell precursors is underway at E12.5, we predicted that *Lhx2* inactivation at this stage would result in the overproduction of one or more of these early born precursors, all previously underproduced after inactivation at E10.5. However, Pou4f+ RGC precursors were overproduced (Fig. 5*C*,*D*) and these other early born cell types were again present in similar or decreased amounts (Fig. 5*E–L*). We quantified these changes by using the $Rosa^{tdTomato}$ (Tomato) recombination reporter (Madisen et al., 2010) to mark the progeny of *Lhx2*-inactivated RPCs and count the number of these Tomato+ cells that went on to express different precursor markers. Thus, we

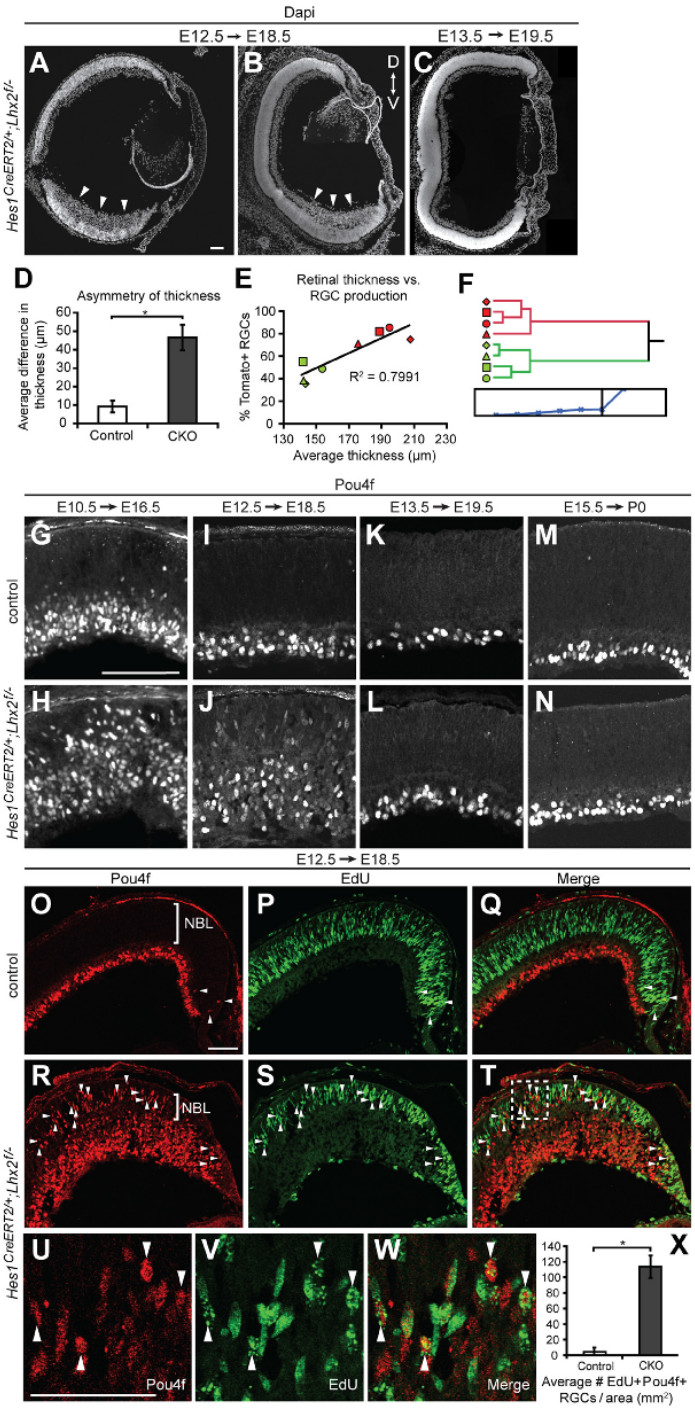

Figure 6. *Lhx2* affects the production of early born cell types during a critical window, and if inactivated during that window, prevents a transition in competence state. ***A–C***, $Hes1^{CreERT2/+};Lhx2^{f/-}$ sections stained with DAPI display an asymmetric phenotype after E12.5 (arrowheads), but not E13.5, inactivation. ***D***, ***E***, This difference in retinal thickness is significant when quantified ($^*p = 0.0059$; $n \geq 3$ for each genotype) and correlates with increased RGC production (different sides of the same retina are indicated with similar shapes). ***F***, Hierarchical clustering also demonstrates the existence of two distinct groups (indicated in color; joining distance at each step is represented on the *x*-axis of the dendrogram and the *y*-axis of the scree plot underneath). ***G–N***, Immunostaining for Pou4f shows that RGC precursor production after inactivation at E12.5 and E13.5 resembles earlier (E10.5) and later (E15.5) phenotypes, respectively. ***O–X***, Immunostaining for Pou4f and detection of EdU shows that RGC precursor production is largely complete at E18.5 in control eyes. In $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes there is a significant number of newly specified RGCs precursors (arrowheads, EdU+Pou4f+) evident in the NBL (***U–W*** show the boxed area in ***T***; $^*p = 0.0108$; $n = 3$ mice for each genotype). Error bars indicate SEM. Scale bars: 100 μm.

determined the relative change in production of different precursors in this population. This revealed a significant increase in the percentage of Pou4f+ RGCs (Fig. 5*M–T*) and reductions in the percentages of Ptf1a+ horizontal/amacrine precursors and Otx2+ photoreceptor precursors. Both Bhlhb5+ and Sox2+ amacrine cell precursors were produced in similar proportion (Fig. 5*U*). To address the possibility that the similarities in neurogenic output between the E10.5 and E12.5 inactivations were due to a delay in Lhx2 downregulation in the E10.5 experiment, we examined Lhx2 expression 48 h after tamoxifen exposure at E10.5 or E12.5. In each case, Lhx2 downregulation was exhaustive (Fig. 5*V–Y*), demonstrating that the similarities in the phenotypes at the two ages were not due to different kinetics of Lhx2 downregulation. These results suggest that RPCs are similar in potential at both E10.5 and E12.5, arguing for a small number of broadly defined competence states and a role for *Lhx2* in ensuring that the different fates available at any given time are produced in the correct proportions.

Lhx2 regulates the RGC competence window

Unexpectedly, *Lhx2* inactivation at E12.5 produced an asymmetric phenotype, obvious through differences in total retinal thickness (Fig. 6*A*,*B*,*D*) that correlated with RGC overproduction (Fig. 6*E*). Analysis of eyes in which axial orientation was maintained revealed that the severely affected region was always located ventrally (Fig. 6*B*). This asymmetry was not due to differential effects on Lhx2 expression since downregulation was largely complete along the extent of the dorsoventral axis within 48 h of tamoxifen exposure (Fig. 5*X*, *Y*). When the two sides of $Hes1^{CreERT2/+};Lhx2^{f/-}$ retinas (separated by the optic nerve head) were treated as individual samples and graphed according to thickness and RGC production, they consistently segregated into two distinct groups (Fig. 6*E*), confirmed by hierarchical clustering analysis (Fig. 6*F*).

Since neurogenesis in the mouse retina initiates dorsally and lags behind in the ventral retina for the first few days (Hufnagel et al., 2010), we suspected that the asymmetric phenotype reflected a temporal shift in which RPCs move away from a dependence on *Lhx2* to limit RGC production. Indeed, the asymmetric phenotype was not observed when tamoxifen treatment was done at E13.5 (Fig. 6*C*), and similar to inactivation at E15.5, the production of RGCs was modestly altered (Fig. 6*G–N*). Since the production of early born cell types including RGCs continues past E13.5, our data reveals that the dependence of RPCs on *Lhx2* for controlling the generation of early born precursors ends before their production windows close.

While these observations suggest that *Lhx2* limits RGC production during an early window of retinal neurogenesis, ectopic RGCs were consistently observed in the NBL at E18.5 in mice treated with tamoxifen at E10.5 or E12.5. Since RGC precursors are initially specified in the NBL, this suggested that RGCs were being generated after the close of their normal production window. To test this, a short-term birth-dating assay was performed: tamoxifen was administered at E12.5 and EdU was administered at E17.5 to mark dividing cells. Retinas were harvested at E18.5 and stained for EdU and Pou4f; double-labeled cells were presumably RGC precursors specified between E17.5 and E18.5. Control retinas never showed more than a few double-labeled cells, confined to the extreme periphery and consistent with the near completion of RGC production (Fig. 6*O–Q*, arrowheads). In contrast, a significant number of newly generated RGC precursors were located throughout the $Hes1^{CreER12/+};Lhx2^{f/-}$ retinas, both centrally and peripherally (Fig. 6*R–X*, arrowheads). Together, these observations reveal a critical period, from approximately E10.5 to E12.5, in which *Lhx2* is required to constrain RGC production. But, if *Lhx2* is inactivated during this critical period, RPCs continue producing RGCs and are less efficient at progressing to the next phase of histogenesis. This latter finding suggests that *Lhx2* contributes to the progression in competence state that restricts the production of early generated cell types and allows for the production of later generated cell types.

Not all RPCs require *Lhx2* to prevent premature differentiation

Conditional inactivation of *Lhx2* consistently led to a depletion of RPCs, yet neu-

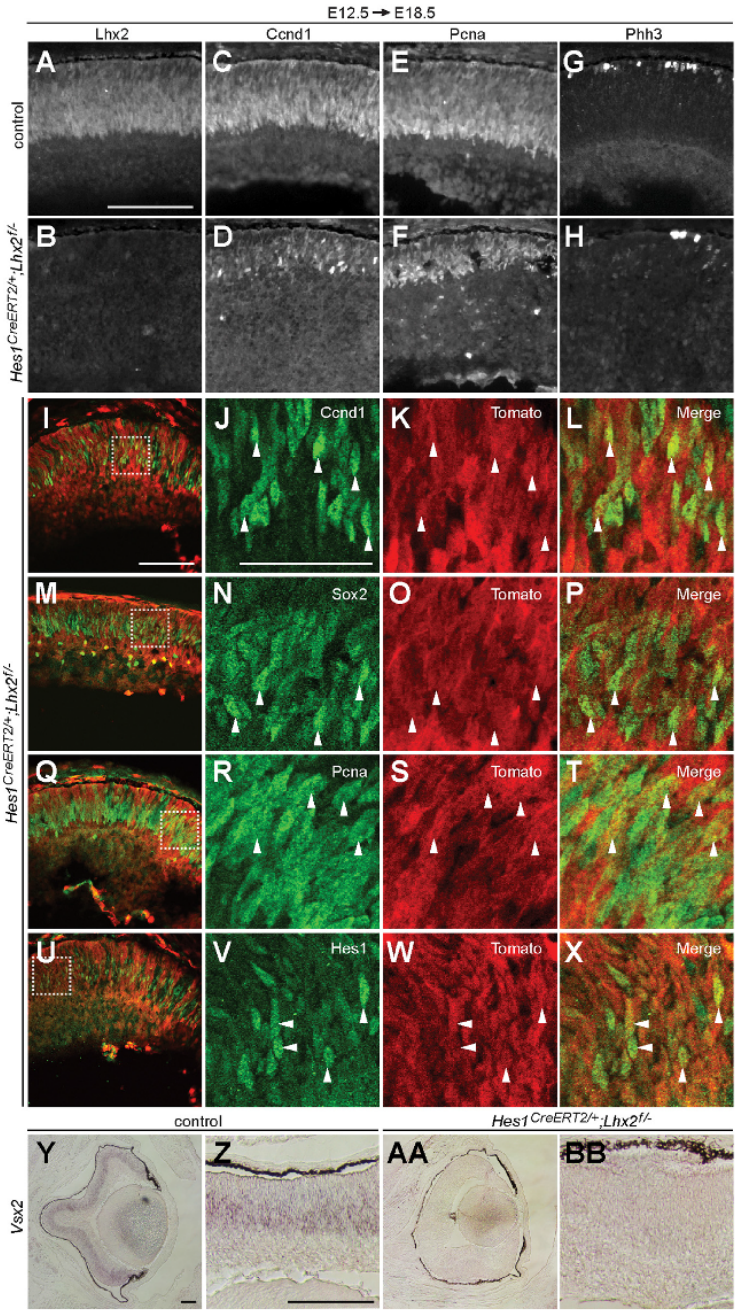

Figure 7. Many progenitors continue to proliferate despite loss of *Lhx2*. ***A–H***, Sections from control and $Hes1^{CreERT2/+};Lhx2^{f/-}$ eyes stained with antibodies against Lhx2 (***A***, ***B***), Ccnd1 (***C***, ***D***), Pcna (***E***, ***F***), and Phh3 (***G***, ***H***) show that while Lhx2 expression is almost completely lost, proliferation markers are still expressed. ***I–X***, Immunostaining for Ccnd1 (***I–L***), Sox2 (***M–P***), Pcna (***Q–T***), and Hes1 (***U–X***) show that *Lhx2*-inactivated cells marked by Tomato expression continue to express both proliferative and progenitor markers (***J–L***, ***N–P***, ***R–T***, and ***V–X*** show boxed areas in ***I***, ***M***, ***Q***, and ***U***, respectively; arrowheads denote examples). ***Y–BB***, RPCs that do remain are still affected, as *Vsx2* expression is completely lost. Scale bars: 100 μm.

rogenesis continued after inactivation (Fig. 6*O–X*), suggesting that some RPCs persisted. It is possible that these RPCs escaped recombination. Alternatively, *Lhx2* may not be used in the same manner in all RPCs. To identify the more likely explanation, we inactivated *Lhx2* at both E10.5 (data not shown) and E12.5 (Fig. 7), in each case examining the RPC population 6 d later. While we observed near-complete loss of Lhx2 (Fig. 7*A*,*B*), reductions in Ccnd1, Pcna, and Phh3 were less severe (Fig. 7*C–H*). This disparity suggested that a cohort of RPCs continued to proliferate despite their loss of *Lhx2*. To directly demonstrate this, we used the Tomato reporter to track recombined RPCs. As expected, several Tomato+ cells in the NBL also expressed markers of proliferation (Ccnd1 and Pcna; Fig. 7*I–L*,*Q–T*) or progenitor status (Sox2 and Hes1; Fig. 7*M–P*, *U–X*). This demonstrates that a subset of RPCs do not require *Lhx2* to maintain their status as proliferating progenitors. Two pieces of evidence suggest, however, that the RPCs remaining after *Lhx2* inactivation were indeed altered. First, expression of the RPC gene *Vsx2* was downregulated throughout the retina (Fig. 7*Y–BB*), consistent with its strong dependence on *Lhx2* activity for its expression in RPCs (Yun et al., 2009). Second, the persistent RPCs produced RGCs after the normal RGC production window closed (Fig. 6*O–X*). Together, these findings argue for the coexistence of at least two cohorts of RPCs that differ in regard to their dependence on *Lhx2* for their maintenance but not for their neurogenic output.

Discussion

The vertebrate retina is a complex and ordered tissue with many constituent cell types, all of which arise from a single population of multipotent progenitor cells. The actions of individual RPCs display surprisingly little order and predictability, yet order is clearly present at the population level. How is this achieved? Though RPCs behave stochastically, this likely occurs within a framework where the number, type, and probability of different outcomes are regulated. Here we have used the inducible *Hes1*^{CreERT2} allele to demonstrate a complex role for *Lhx2* in regulating this framework by promoting RPC maintenance, regulating the likelihood of competing or alternative fate choices, and allowing for a shift in competence state.

At every age examined, the conditional inactivation of *Lhx2* resulted in a significant depletion of the RPC population and an increase in neurogenesis. This suggested that *Lhx2* normally serves to increase the probability of self-renewal, resulting in maintenance of the RPC population. However, this depletion was never complete: many *Lhx2*-inactivated RPCs continued to proliferate and express progenitor markers well after inactivation. This suggested the presence of more than one cohort of RPCs, differing in regard to their requirement for *Lhx2*. Such a scenario may be explained by *Lhx2*-dependent and *Lhx2*-independent cohorts of RPCs that coexist but develop in parallel. Another possibility is that *Lhx2* has multiple roles in RPCs at different times during their normal developmental progression. We favor the latter possibility, as our results show that RPCs that do not immediately exit the cell cycle are still affected in terms of gene expression and neurogenic output. This is also consistent with our previous work examining *Ccnd1*$^{-/-}$ mutant mice, which demonstrated a significant yet incomplete depletion of RPCs and selective overproduction of RGCs (Das et al., 2009). Both *Ccnd1*$^{-/-}$ and *Lhx2* conditional inactivation phenotypes suggest the presence of distinct steps in RPC progression. Thus, we propose the following model (Fig. 8): at any given time, a subset of RPCs are specified to differentiate on the basis of stochastic differences and/or signals received. These RPCs then become limited in proliferative capacity and biased toward the generation of certain cell types, reflecting their competence at the time. The specification of these "biased" RPCs is an iterative process, such that as they exit the cell cycle,

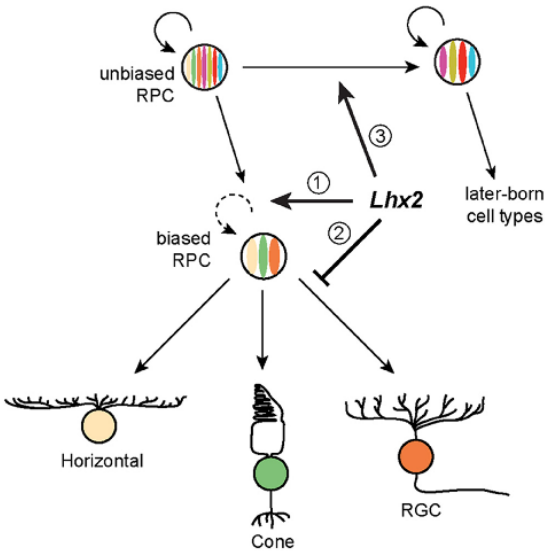

Figure 8. Model of *Lhx2* function in RPCs. While all RPCs are presumably competent to undergo differentiation, some RPCs do so on the basis of stochastic differences and/or signals received, and become biased toward both neurogenic divisions as well as the production of certain fates (a reflection of their competence at the time). We hypothesize that *Lhx2* acts within this set of biased RPCs to not only increase the probability of proliferative divisions (arrow 1), but to ensure that different precursors are produced in the correct proportions by limiting the production of RGC precursors (arrow 2). This process is likely to be reiterated later in retinal development. In addition, *Lhx2* impacts unbiased RPCs by regulating competence progression during a limited temporal window (before ~E13.5; arrow 3), and possibly proliferation through its regulation of *Vsx2* and other factors yet to be identified. Colors in RPCs depict their potential to generate fated precursors.

they are replaced from an upper pool of "unbiased" RPCs. Our findings suggest that RPCs that require *Lhx2* or *Ccnd1* for their immediate maintenance may represent cohorts of this biased RPC population (Fig. 8, arrow 1). There is precedence for such a model of progenitor progression both in the retina (Brzezinski et al., 2011; Hafler et al., 2012) and elsewhere, as biased RPCs in our model are analogous to both intermediate neural progenitors generated from stem-like radial glia in the cortex (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004) as well as ganglion mother cells generated from *Drosophila* neuroblasts (Isshiki et al., 2001).

Inactivation of *Lhx2* during both early and late phases of neurogenesis resulted in the selective overproduction of certain cell types, occurring at the expense of others. This suggested that RPCs are competent to generate more than one cell type at a time, an idea supported by the observation of discordant two-cell clones in many different lineage studies (Turner and Cepko, 1987; Wong and Rapaport, 2009; Brzezinski et al., 2011; Hafler et al., 2012). However, RPCs do not behave randomly when choosing between fates—some are chosen more often than others. Mechanisms must exist to provide bias, and our results suggest that *Lhx2* is a key player in such a mechanism(s) (Fig. 8, arrow 2). Accordingly, both negative and positive feedback signals affect the generation of specific cell types and promote diversity (Reh and Tully, 1986; Altshuler and Cepko, 1992; Watanabe and Raff, 1992; Belliveau and Cepko, 1999; Kim et al., 2005; Wang et al., 2005). Notch signaling introduces differences between otherwise equivalent cells, and unique components of the pathway (ligands, receptors, effectors) regulate the generation of specific cell types (Jadhav et al., 2006; Yaron et al., 2006; Riesenberg et al., 2009). Asymmetric inheritance of the Notch antagonist *Numb* acts generally to promote asymmetric terminal divisions, with

daughter cells assuming different fates (Kechad et al., 2012). *Lhx2* may regulate diversity through interaction with one or more of these pathways, as loss of *Lhx2* reduces the relative probability of assuming different fates.

In addition to demonstrating that *Lhx2* regulates the generation of distinct cell types during early and late phases of neurogenesis, temporal analysis allowed us to locate the approximate time at which this change in regulation occurs. The different phenotypes seen after earlier (E10.5, E12.5) versus later (E13.5, E15.5) inactivations are indicative of a normal shift in competence among the RPC population. Early loss of *Lhx2* affects competence by preventing this transition, yet later inactivation does not. And while certain cell types are selectively overproduced in both cases, the other cell types are not completely lost. Thus, *Lhx2* regulates the shift between competence states rather than actually conferring and/or limiting competence (Fig. 8, arrow 3). How *Lhx2* does this is not yet clear. One possibility is that it regulates the expression of *Ikaros*, a *Hunchback* ortholog that confers competence to RPCs to generate early born cell types (Elliott et al., 2008). Other possibilities are that *Lhx2* interacts with the Dicer or Sonic Hedgehog (Shh) pathways. The *Dicer* and *Shh* conditional mutants (Wang et al., 2005; Georgi and Reh, 2010) produced phenotypes similar to *Lhx2* inactivations initiated at E12.5 and earlier. As *Shh* also operates as a negative feedback signal for controlling RGC precursor production (Zhang and Yang, 2001; Wang et al., 2005; Sakagami et al., 2009), *Lhx2* may regulate neurogenic output and competence progression through interaction with a single pathway.

Lhx2 acts as a selector gene necessary for autonomous specification of regional identity in the cortex (Bulchand et al., 2001; Mangale et al., 2008; Chou et al., 2009), and similar roles may exist in the developing amygdala and pituitary (Remedios et al., 2004; Zhao et al., 2010). Correspondingly, *Lhx2* participates in eye-field formation (Zuber et al., 2003; Tétreault et al., 2009; Hägglund et al., 2011) and is required for regionalization of the optic vesicle through cell-autonomous regulation of gene expression (Yun et al., 2009). At a cellular level, the function of *Lhx2* is context dependent. In the olfactory epithelium, hippocampus, and thalamus *Lhx2* promotes neurogenesis as well as the maturation and axonal outgrowth of postmitotic neurons (Hirota and Mombaerts, 2004; Kolterud et al., 2004; Lakhina et al., 2007; Saha et al., 2007; Peukert et al., 2011; Sanuki et al., 2011; Subramanian et al., 2011; Berghard et al., 2012; Marcos-Mondéjar et al., 2012). In the cortex, hematopoietic lineage, and hair follicle, *Lhx2* promotes progenitor and stem-cell maintenance, preventing premature differentiation (Rhee et al., 2006; Dahl et al., 2008; Kitajima et al., 2011; Chou and O'Leary, 2013). In addition to cellular context, several of these functions are limited to a critical period and thus very dependent on timing (Mangale et al., 2008; Chou et al., 2009; Subramanian et al., 2011).

In the present study we have demonstrated that *Lhx2* is required for the maintenance of RPCs during retinal neurogenesis, consistent with a role in stem and/or progenitor cell maintenance and self-renewal. Importantly, the ability to induce inactivation at multiple time points has allowed us to gain additional insight into the function of *Lhx2*, and our results are significant in several different regards: First, we have reported a *selective* role for *Lhx2* in progenitor maintenance, indicative of heterogeneity within the progenitor population. Second, we have reported a function for *Lhx2* in regulating neuronal *diversity* rather than the general promotion or prevention of neurogenesis. Last, we have reported a function for *Lhx2* in regulating competence progression, a fundamental property of progenitors that allows for ordered cell production in multiple tissues. In line with *Lhx2*'s requirements in promoting early eye development, these results position *Lhx2* as an intrinsic factor essential for coordinating multiple aspects of retinal development.

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CHAPTER 3

PROGENITOR CELL DYNAMICS IN THE EMBRYONIC RETINA AS REVEALED BY GENETIC MANIPULATION OF LHX2 AND RPBJ

Abstract

Determining how cells progress through the distinct steps of a given lineage is a fundamental question in developmental biology. In the retina, recent studies have begun to describe a diverse class of biased, intermediate progenitors, and identifying the mechanisms that control the generation and proliferation of different RPC subtypes will thus be crucial for understanding how retinal histogenesis is coordinated. Here, we inactivate the Notch transcription factor *Rbpj*, both in isolation and combination with *Lhx2*, demonstrating that these factors are required in succession for the maintenance and/or proliferation of RPCs at distinct stages in their lineage progression.

Introduction

In vertebrates, all of the major retinal cell types are produced in a distinct yet overlapping order from a single pool of retinal progenitor cells (RPCs); thus, a sufficient population of RPCs must be maintained throughout neurogenesis to ensure that each cell type is generated in both the correct number and proportion. *Lhx2* is an essential regulator of early optic patterning and morphogenesis (Yun et al. 2009; Porter et al. 1997), expressed in most, if not all, embryonic RPCs, and whose conditional inactivation results in a premature depletion of the RPC pool and corresponding increase in neurogenesis (Gordon et al. 2013). Despite near-absolute loss of *Lhx2*, however, this depletion is incomplete, with many RPCs continuing to proliferate and give rise to neuronal precursors well after inactivation. This underscores the heterogeneity of the RPC population, and suggests that separable cohorts of RPCs differ in their requirement for *Lhx2*. Identifying the basis for this difference, as well as other intrinsic factors that may

sustain proliferation in the absence of *Lhx2*, will provide insight into how the RPC population is organized.

The Notch signaling pathway is associated with maintenance of stem and progenitor cell populations throughout many developing tissues, and in the cortex, may inhibit the formation of intermediate neural progenitors (INPs) through negative feedback (Mizutani et al. 2007; Gao et al. 2009; Imayoshi et al. 2010; Nelson et al. 2013). We hypothesize that a similar scenario exists in the retina, with *Lhx2* responsible for maintaining a pool of Notch-independent, intermediate progenitors that are limited in proliferative capacity and close to cell-cycle exit - referred to here as biased RPCs. In turn, Notch activity in upstream progenitors is likely responsible for limiting the generation of biased RPCs via negative feedback, ultimately maintaining two major RPC pools in a state of dynamic equilibrium. Here, we address this issue by examining progenitor depletion and neurogenic output after combinatorial inactivation of *Lhx2* and the Notch transcription factor *Rbpj*.

Results and discussion

To enable an accurate comparison with our previous work (Gordon et al. 2013), we conditionally inactivated floxed alleles of *Lhx2* (Mangale et al. 2008) and *Rbpj* (Han et al. 2002) with the inducible $Hes1^{CreERT2}$ driver (Kopinke et al. 2011). $R26R^{EYFP}$ (Srinivas et al. 2001) was used to follow inactivated RPCs. After administration of a single dose of tamoxifen (TM), only a small number of EYFP-negative radial clones, representing non-recombined cells, remained (Fig. 3.1A, B). Lamination was severely disrupted in the retinas of *Rbpj* conditional knock-out (CKO) mice when compared to control tissue, consistent with previous Notch loss-of-function studies (Tomita et al.

1996; Rocha et al. 2009; Jadhav et al. 2006; Yaron et al. 2006; Riesenberg et al. 2009; Zheng et al. 2009) (Fig. 3.1C, D). Differentiated cells often failed to accumulate on the basal side of the retina, and instead were often found apically (Fig. 3.1D, arrow). Importantly, *Lhx2* expression was maintained in *Rbpj* CKO retinas (Fig. 3.1E, F). Continued expression of *Hes5,* as well as a lack of rosette formation in *Lhx2* CKO retinas (see below) were indicative of continued Notch pathway activity. *Hes1* expression did decrease after loss of *Lhx2* (Gordon et al. 2013), but other signaling pathways converge on *Hes1* (Wall et al. 2009; Hashimoto et al. 2006), and compromising their activity may have contributed to its downregulation.

Progenitor maintenance

Prior studies describing *Rbpj* loss-of-function phenotypes in the developing retina showed a reduction of the RPC pool as well as decreased proliferation (Riesenberg et al. 2009; Zheng et al. 2009). To compare the level of RPC depletion in *Rbpj* CKO and *Lhx2;Rbpj* double-CKO (DCKO) retinas to the *Lhx2* CKO phenotype, we used the same paradigm for TM treatment (E12.5) and analysis (E18.5) (Gordon et al. 2013). Analyses of DCKO eyes were limited to the ventral retina, where we previously demonstrated that the effects of *Lhx2* inactivation were most pronounced (Gordon et al. 2013). Phosphohistone H3-positive ($pHH3^+$) RPCs, normally localized to the apical side of the retina (Fig. 3.1G, bracket), were found in the center of rosettes in *Rbpj* CKO retinas (Fig. 3.1H). Cyclin D1 (Ccnd1) and Proliferating Cell Nuclear Antigen (PCNA), comprehensive RPC cell cycle markers (Barton and Levine 2008), were decreased in *Rbpj* CKO retinas (Fig. 3.1I-L), though several $EYFP^+$ cells continued to express these markers, as well as pHH3 (Fig. 3.2). Similar to *Lhx2*, these observations indicate that

Rbpj is required for progenitor maintenance and/or proliferation in a subset of RPCs, even though both factors are expressed in most, if not all, embryonic RPCs (Maurer et al. 2014; Gordon et al. 2013).

Since *Lhx2* is still expressed in the *Rbpj* CKO retina and Notch signaling persists in the *Lhx2* CKO retina, it stood to reason that *Lhx2* and *Rbpj* may regulate progenitor maintenance in different pools of RPCs. Indeed, significantly fewer $pHH3^+$, $Ccnd1^+$, and $PCNA^+$ cells were present in DCKO retinas (Fig. 3.1M-O) than *Rbpj* CKO (Fig. 3.1H, J, L) or *Lhx2* CKO retinas (Gordon et al., 2013). A small cohort of $EYFP^+$ cells still expressed these different proliferative markers (Fig. 3.2), and it is possible that they retained some level of Lhx2 and/or Rbpj activity. Alternatively, these cells may not have required *Lhx2* and *Rbpj* for their continued proliferation, invoking the possibility of additional maintenance factors. Regardless, our data support the hypothesis that *Lhx2* and *Rbpj* are essential for progenitor maintenance in large yet separable cohorts of embryonic RPCs.

RPC gene expression

Our hypothesis predicted that loss of *Rbpj* or *Lhx2* should result in an increase or decrease, respectively, of biased RPC markers. In addition, any decrease in this biased RPC population observed in *Lhx2* CKO retinas may eventually be corrected for, through negative feedback, with the two RPC cohorts establishing a new equilibrium. We administered TM at E12.5 and performed analyses at E14.5, E16.5, and E18.5. While there is no comprehensive marker for this biased RPC population, several genes mark RPCs with limited proliferative capacity, including *Ascl1*, *Olig2*, *Ngn2*, *Dll1*, and *Dll4* (Hafler et al. 2012; Brzezinski et al. 2011; Rocha et al. 2009; Nelson et al. 2009). Of

these, we chose to examine *Ngn2*, *Dll1*, and *Dll4*, as all three are expressed very shortly before cell-cycle exit and appear to mark biased RPCs in a general sense, with relatively little fate bias (Brzezinski et al. 2011; Rocha et al. 2009). We also examined *Vsx2* and *Pax6* as comprehensive RPC markers, as well as *Hes1* and *Hes5* as Notch pathway read-outs.

At E14.5, biased RPC markers were unaffected in both *Lhx2* and *Rbpj* CKO retinas (Fig. 3.3). Consistent with our previous findings, however, *Hes1* expression was decreased and *Vsx2* expression lost in *Lhx2* CKO retinas (Fig. 3.4A, B, I, and J) (Gordon et al. 2013; Yun et al. 2009). In addition, *Hes1* expression was expectedly decreased in *Rbpj* CKO retinas (Fig. 3.4K, L).

At E16.5, biased RPC markers were unaffected in *Lhx2* CKO retinas (Fig. 3.5). In *Rbpj* CKO retinas, however, *Ngn2* expression increased, with *Ngn2*$^{+}$ cells clustering together radially (Fig. 3.5C, D, arrowheads). *Dll1* expression also increased, and surprisingly, *Dll4* expression appeared to decrease slightly (Fig. 3.5). *Vsx2* was still absent in *Lhx2* CKO retinas, and expressed in *Rbpj* CKO retinas (Fig. 3.6). *Hes1* was decreased in *Lhx2* CKO retinas, while *Hes1* and *Hes5*, surprisingly, showed only a slight decrease after the loss of *Rbpj*. This suggested to us that rather than serving as exclusive read-outs of the Notch pathway, these genes may be under more complex regulation (Fig. 3.6). This issue is highlighted by previously differing reports on the expression of *Hes5* in *Rbpj* CKO retinas (Riesenberg et al. 2009; Zheng et al. 2009), and potentially explained by 1) compensation between *Hes* genes, 2) *Rbpj*-independent Notch signaling, or 3) Notch-independent expression of *Hes1* and/or *Hes5*, all possibilities supported at some

level by the work of others (Hatakeyama et al. 2004; Wall et al. 2009; Hashimoto et al. 2006; Andersen et al. 2012).

At E18.5 *Lhx2* CKO retinas displayed a thickened differentiated cell layer (DCL) and *Rbpj* CKO retinas contained characteristic rosettes (Fig. 3.7C, D, E, and F). Relative to the approximate size of the neuroblast layer (NBL), where RPCs reside, *Ngn2* and *Dll1* expression was still maintained in *Lhx2* CKO retinas (Fig. 3.7C, D); thus, we were unable to detect a clear loss of these biased RPC markers in *Lhx2* CKO retinas at the ages examined. However, one possible explanation for this result may be that the decrease was simply missed by our analysis, as the resulting loss of negative feedback would be presumed to quickly restore the population to a new equilibrium. In contrast, we did observe a clear, and predicted, increase of *Ngn2* and *Dll1* expression in *Rbpj* CKO retinas (Fig. 3.7E, F). *Vsx2* expression was again absent from *Lhx2* CKO retinas and present in *Rbpj* CKO retinas (Fig. 3.8A-D). *Hes1* expression was difficult to detect (Fig. 3.8I-L), while *Hes5* expression decreased in both *Lhx2* CKO and *Rbpj* CKO retinas (Fig. 3.8M-P).

We also examined the expression of these genes in DCKO retinas at E18.5. In contrast to the strong increase observed after *Rbpj* CKO, *Ngn2* and *Dll1* expression levels clearly decreased (Fig. 3.7G, H). This suggests that the additional removal of *Lhx2* in the DCKO retina prevented an ectopic build-up or overproduction of biased RPCs, implying that *Lhx2* is necessary for their maintenance in proliferation in some manner and supporting the possibility that the lack of any significant effect observed in *Lhx2* CKO retinas was perhaps due to experimental timing. *Vsx2* expression was absent, in accordance with loss of *Lhx2* (Fig. 3.8E, F). *Hes1* expression was again difficult to detect

(Fig. 3.8Q, R), but *Hes5* expression was decreased, similar to both *Lhx2* CKO and *Rbpj* CKO (Fig. 3.8W, X).

Neurogenic output

Previously, we characterized neurogenic output in the *Lhx2* CKO retina using five different markers: Pou4f, marking RGC precursors, Otx2, marking both photoreceptor and bipolar cell precursors (Nishida et al. 2003), Bhlhb5 and Sox2, marking subsets of the amacrine cell population (Taranova et al. 2006; Feng et al. 2006), and Ptf1a, marking both horizontal and amacrine cell precursors (Fujitani et al. 2006). RGCs were selectively overproduced, with other early-born cell types unchanging or decreased in number (Gordon et al. 2013). To characterize the neurogenic output of *Rbpj* CKO and DCKO retinas, we performed an identical analysis. Consistent with previous studies (Riesenberg et al. 2009; Zheng et al. 2009), and in comparison to controls (Fig. 3.9A), RGC production increased in *Rbpj* CKO retinas (Fig. 3.9B). A similar, but more pronounced effect was observed in DCKO retinas (Fig. 3.9C, P). Photoreceptor production, in comparison to controls (Fig. 3.9D), increased dramatically in *Rbpj* CKO retinas (Fig. 3.9E). Quantifying the production of RXRγ^{+} cells located within rosettes confirmed that this overproduction was due at least in part to an overproduction of cones (Fig. 3.10F, G, L). This overproduction of photoreceptor precursors was lost in DCKO retinas (Fig. 3.9F), with production returning to wild-type levels (Fig. 3.9P).

This result was interesting for two reasons: First, it suggested that the RGC overproduction observed in *Lhx2* CKO retinas did not occur at the direct expense of photoreceptors, by demonstrating that their underproduction is not a prerequisite for the corresponding RGC overproduction. Thus, RPCs may not typically be faced with

deciding directly between RGC and photoreceptor fates. Rather, RPCs that give rise to these cell types may instead exist as two distinct populations, or simply occupy different competence states. Second, this result highlighted the importance of careful interpretation. Together, and assuming no overlap, our five separate markers accounted for approximately 90% of the recombined ($EYFP^+$) population in *Rbpj* CKO retinas (Fig. 3.10L). However, in DCKO retinas, they only accounted for approximately 60% of the recombined ($EYFP^+$) population (Fig. 3.10M). This is counterintuitive, as RPC depletion is more severe in these eyes, and should elevate the percentages of postmitotic cells in the $EYFP^+$ population. At least two possible scenarios may explain this: First, $EYFP^+$ cells in the DCKO retina may have assumed a postmitotic fate, or quiescent status, not recognized by the markers used in this study. Second, *Otx2* may have been prematurely expressed in RPCs within the *Rbpj* CKO retina, leading us to inadvertently classify $Otx2^+$ RPCs as photoreceptors. This possibility seems more likely, as Notch signaling may directly regulate Otx2 transcription (Muranishi et al. 2011). Further removal of *Lhx2* in the DCKO retina is unlikely to restore Notch signaling, and thus while Otx2 regulation was probably similarly disturbed, it may have gone unnoticed due to the fact that RPC depletion was more complete, with $Otx2^+$ RPCs potentially exiting the cell-cycle faster and avoiding misclassification.

In addition to the effect observed on RGCs and photoreceptors, changes were also seen in the production of horizontal and amacrine cell precursors. Bhlhb5, Sox2, and Ptf1a, all expressed control retinas (Fig. 3.9G, J, M), were almost completely lost in *Rbpj* CKO retinas (Fig. 3.9H, K, N). *Rbpj* functions in a Notch-independent manner as part of the PTF1 complex necessary for the specification of GABAergic interneurons in the

developing spinal cord (Hori et al. 2008), and these results suggest a similar function may exist in the retina, as recently described in chicks (Lelièvre et al. 2011).

We have shown that *Lhx2* and *Rbpj* work in a complementary manner to regulate RPC proliferation and act at distinct stages in the lineage progression of these cells. In addition, our examination of neurogenic output in both *Rbpj* CKO and DCKO retinas supports multiple roles for *Rbpj* in the regulation of fate decisions – separable from the role of Notch signaling in RPC maintenance. Both these, as well as analogous and separable functions of *Lhx2*, are represented in our model of early-stage retinal neurogenesis (Fig. 3.11). Our previous work supports this model, in which an upper pool of unbiased RPCs continuously generates a lower pool of more limited RPCs (Gordon et al. 2013; Das et al. 2009). Further, other groups have identified analogous RPC subtypes in which terminal fate bias is reflected through gene expression (Hafler et al. 2012; Wang et al. 2014; Emerson et al. 2013; Godinho et al. 2007; Suzuki et al. 2013). Defining the entire range of biased RPC subtypes, and determining how their relative production is controlled and coordinated, represents one of the major challenges for retinal development. While we demonstrate here that Notch activity functions in a general manner to limit their production, the regulatory relationships between both Notch pathway components, bHLH genes, and other biasing factors are complex (Maurer et al. 2014; Nelson et al. 2009), and future studies examining individual components and relationships will be required. Furthermore, it will also be important to determine exactly how *Lhx2* affects proliferation in a selective manner.

Materials and methods

Animals

The *Lhx2* conditional allele was generated by Mangale et al. and genotyped as described. The *Rbpj* conditional allele was generated by Han et al. and genotyped using the following primers: floxed: F-GTTCTTAACCTGTTGGTCGGAACC, R-CTAGAACAGGCTGCCTGATCACCC, deletion: F-CAAAGCCCCTTTCTTT-GTGCGTGCC, R-GCTTGAGGCTTGATGTTCTGTATTGC. The $Hes1^{CreERT2}$ knock-in allele was generated by Kopinke et al. and genotyped as described. The $R26R^{EYFP}$ allele was generated by Srinivas et al. and genotyped as described. For *Lhx2* CKO and *Rbpj* CKO, mutant animals harbored a floxed allele and deletion at the appropriate locus (Ex: $Hes1^{CreERT2/+};Lhx2^{f/-};R26R^{EYFP/+}$). Control littermates differed only in that the wild-type allele was present in place of the deletion (Ex: $Hes1^{CreERT2/+};Lhx2^{f/+};R26R^{EYFP/+}$). For DCKO, mutant animals harbored a floxed allele and deletion at each locus ($Hes1^{CreERT2/+};Lhx2^{f/-};Rbpj^{f/-};R26R^{EYFP/+}$), and control littermates had a wild-type allele at each locus ($Hes1^{CreERT2/+};Lhx2^{f/+};Rbpj^{f/+};R26R^{EYFP/+}$). Embryonic age determinations were based on plug date and morphological criteria. All animal use and care was conducted in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee and set forth in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals. Efforts were made to minimize discomfort to animals and when possible, the number of animals needed per analysis was kept to a minimum.

Administration of TM

TM (Sigma T5648) was dissolved in corn oil (Sigma C8267) at a concentration of 20 mg/ml and 0.2 mg/g body weight was administered to pregnant dams by oral gavage at E12.5 with 22G 1.5 inch feeding needle.

Immunohistochemistry and *in situ* hybridization

Embryo heads or eyes were dissected in PBS and fixed in 4% PFA for 30 minutes at room temperature. Tissue was washed with PBS, put through a gradient of sucrose solutions, embedded in OCT (Sakura Finetek, Torrance, CA), and stored at -80°C. Frozen tissues were sectioned on a cryostat at a thickness of 12 μm.

Primary antibodies used were: rabbit anti-LHX2 (Edwin Monuki, University of California, Irvine, CA; 1:50), goat anti-GFP (Rockland; 1:5000), rabbit anti-GFP (Abcam, 1:4000), rabbit anti-CCND1 (Lab Vision; 1:400), mouse anti-PCNA (DAKO; 1:500), rabbit anti-pHH3 (Upstate Biotechnology; 1:500), goat anti-POU4F (Santa Cruz; 1:50), rabbit anti-SOX2 (Abcam; 1:400), anti-RXRγ (Santa Cruz; 1:200), rabbit anti-NR2E3 (Anand Swaroop, National Eye Institute, Bethesda, MD; 1:100), goat anti-BHLHB5 (Santa Cruz; 1:1000), guinea pig anti-PTF1A (Jane Johnson, University of Texas Southwestern, Dallas, TX; 1:8,000), and rabbit anti-OTX2 (Chemicon; 1:15,000). Primary antibodies were followed with species-specific secondary antibodies conjugated to Alexa Fluor 488, 568, or 647 (Invitrogen/Molecular Probes, Eugene, OR). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Fluka). Panels showing fluorescence-based protein detection are single scan confocal images obtained with a Fluoview 1000 confocal microscope (Olympus).

In situ hybridization was performed as previously described (Schaeren-Wiemers and Gerfin-Moser 1993). Probes used in this study were digoxigenin-labeled anti-sense probes against *Hes1*, *Hes5*, *Vsx2*, *Pax6*, *Ngn2*, *Dll1*, and *Dll4*.

Quantification and statistical analyses

For lineage tracing experiments, different cell types were identified based on the expression of precursor markers. The percentage of the recombined population that assumed a particular fate was calculated as the number of EYFP+, Marker+ cells over total EYFP+ cells. For all quantifications, at least three animals of each genotype were used, across at least two different litters. For determining significance in all comparisons we used an alpha level of 0.05 and a two-sided Aspin-Welch-Satterthwaite-Student's t-test, which assumes normal distribution but unequal variance. JMP Pro 11.0 software was used for all calculations, and all data graphed are shown as the mean ± standard error.

Figure 3.1. RPC Depletion is more severe in DCKO retinas than in *Rbpj* CKO retinas. (A, B) *Hes1*^{CreERT2} drove recombination in the vast majority of RPCs, indicated by EYFP. (C, D) *Rbpj* CKO retinas (D) contained rosettes and differentiating cells inappropriately located on the apical side of the retina (arrow), in comparison to controls (C). (E, F) Lhx2 was expressed in both control (E) and *Rbpj* CKO retinas (F), with any reduction in the number of positive cells likely occurring as a result of RPC depletion. (G-O) Proliferation was decreased in *Rbpj* CKO retinas, and markedly worse in DCKO retinas, when compared to controls and assessed with the markers pHH3 (G, H, M), Ccnd1 (I, J, N), and Pcna (K, L, O). Scale bar: 100 μm.

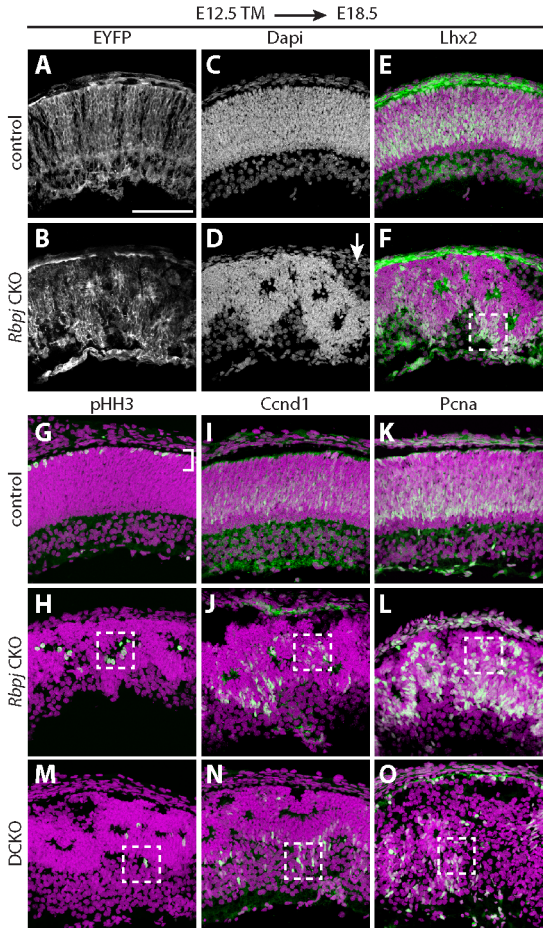

E12.5 TM ⟶ E18.5
EYFP
Dapi
Lhx2
A
C
E
control
B
D
F
Rbpj CKO
pHH3
Ccnd1
Pcna
G
I
K
control
H
J
L
Rbpj CKO
M
N
O
DCKO

Figure 3.2. EYFP is co-expressed with markers of proliferation in *Rbpj* CKO and DCKO retinas. (A-C) Expression of pHH3 (A), Ccnd1 (B), and Pcna (C) is similar in controls from both *Rbpj* CKO (here) and DCKO crosses (Fig. 1). (D, E) DCKO retinas show both rosettes (indicative of Notch loss-of-function) and a ventral thickening of the DCL (as observed with *Lhx2* CKO retinas in our previous study) (E), compared to control retinas (D). (F') High magnification insets confirmed that Lhx2 was expressed in EYFP-positive (i.e. Rbpj-negative) cells (arrowheads point to examples) in *Rbpj* CKO retinas. (H', K', N') Insets corresponding to panels in Fig. 1 show that some proliferating cells that co-expressed EYFP (arrowheads) in *Rbpj* CKO retinas. (I', L', O') Similar examples were found, though less abundant, in DCKO retinas (arrowheads). Scale bars: *A*, 100 μm; F', 20 μm.

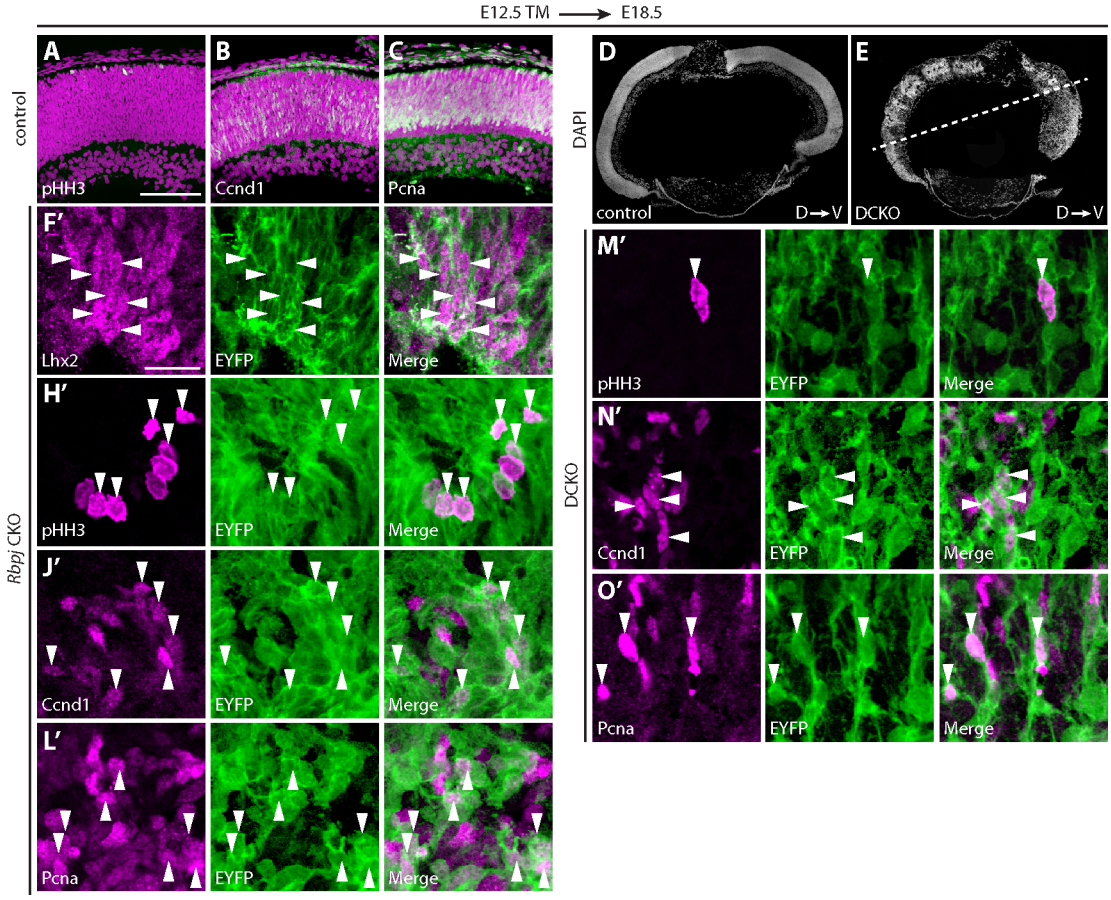

E12.5 TM
E18.5
control
Rbpj CKO
DAPI
DCKO
A
B
C
D
E
pHH3
Ccnd1
Pcna
control
DCKO
D→V
F′
H′
J′
L′
M′
N′
O′
Lhx2
EYFP
Merge

Figure 3.3. Analysis of biased RPC markers at E14.5. (A-D) *Ngn2* was strongly expressed in a subset of RPCs, and expression did not change in either *Lhx2* CKO (B) or *Rbpj* CKO (D) retinas relative to controls (A, C). (E-H) *Dll4* was expressed in a subset of RPCs, and expression did not change in either *Lhx2* CKO (F) or *Rbpj* CKO (H) retinas relative to controls (E, G). (I-L) *Dll1* was expressed in a subset of RPCs, and again, expression did not change in *Lhx2* CKO (J) or *Rbpj* CKO (L) retinas relative to controls (I, K). Scale bars: 100 μm.

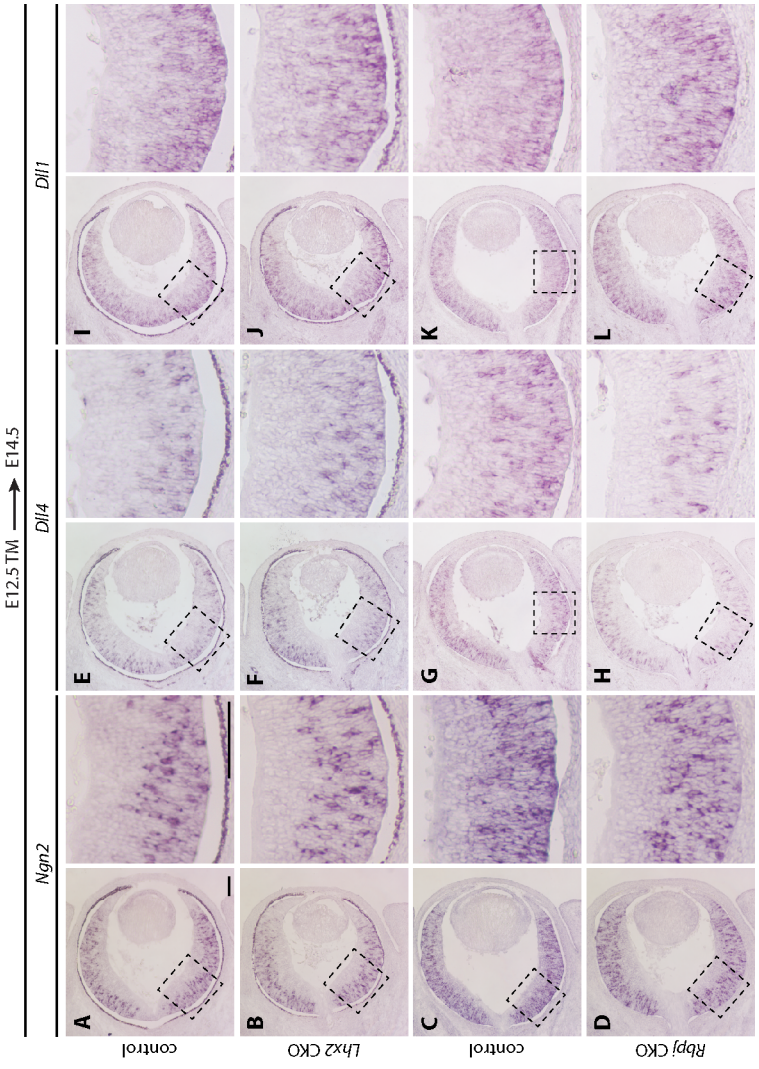

E12.5 TM
E14.5
Ngn2
Dll4
Dll1
A
B
C
D
E
F
G
H
I
J
K
L
control
Lhx2 CKO
control
Rbpj CKO

Figure 3.4. Analysis of general RPC markers at E14.5. (A-D) *Vsx2* was strongly expressed in most, if not all, RPCs of control retinas (A, C), yet absent in *Lhx2* CKO retinas (B), consistent with our previous work (Gordon et al. 2013; Yun et al. 2009), and slightly decreased in *Rbpj* CKO retinas (D). (*E-H*) *Pax6* showed no change in expression across control (E, G), *Lhx2* CKO (F), and *Rbpj* CKO (H) retinas. (I-L) *Hes1* expression was decreased, though not lost, in both *Lhx2* CKO (J) and *Rbpj* CKO (L) retinas. (M-P) *Hes5* expression did not change across control (M, O), *Lhx2* CKO (N), and *Rbpj* CKO (P) retinas. Scale bars: 100 μm.

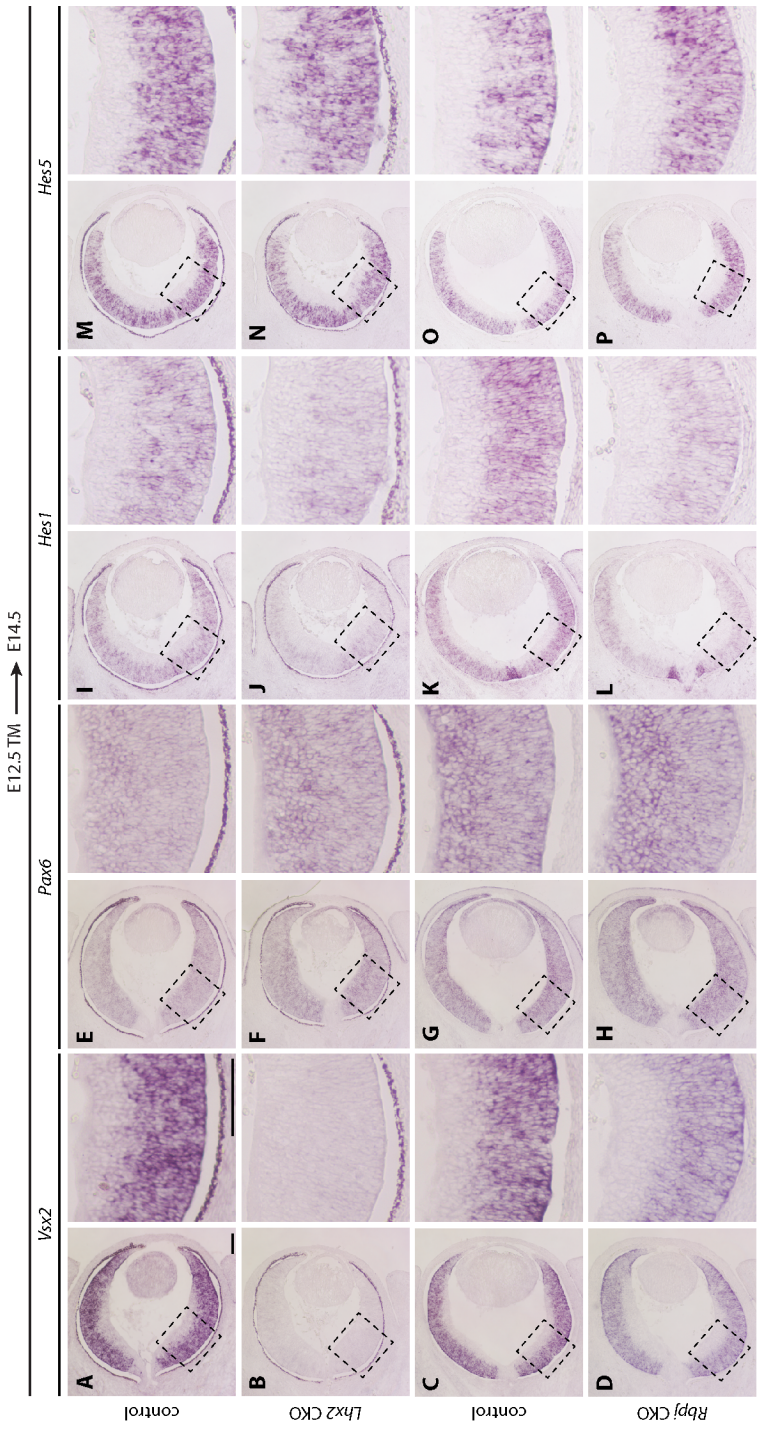

E12.5 TM
E14.5
Vsx2
Pax6
Hes1
Hes5
control
Lhx2 CKO
control
Rbpj CKO
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 3.5. Analysis of biased RPC markers at E16.5. (A-D) *Ngn2* expression did not change in *Lhx2* CKO retinas (B), yet increased slightly in *Rbpj* CKO retinas (D), with cells expressing *Ngn2* clustered together regularly and in a radial manner (arrowheads). (E-H) *Dll4* expression was weak, yet no different, in control (E, G), *Lhx2* CKO (F), and *Rbpj* CKO (H) retinas. (I-L) Similarly, no significant changes in *Dll1* expression were observed across control (I, K), *Lhx2* CKO (J), and *Rbpj* CKO (L) retinas. Scale bars: 100 μm.

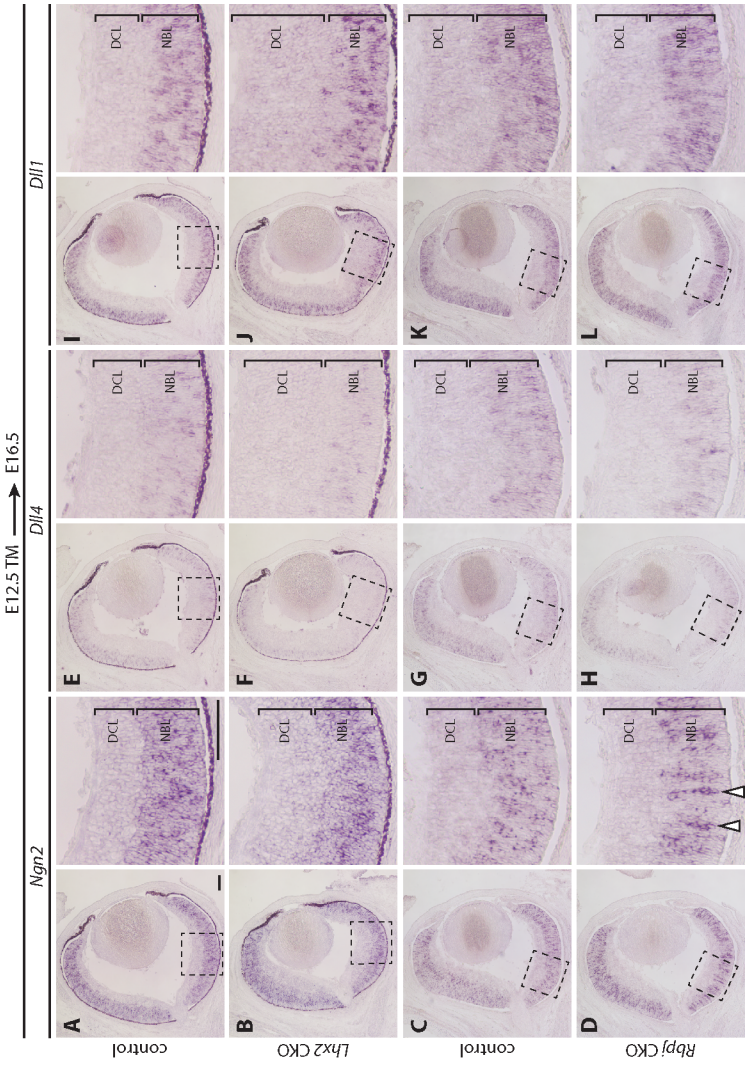

E12.5 TM
E16.5
Ngn2
Dll4
Dll1
control
Lhx2 CKO
control
Rbpj CKO
A
B
C
D
E
F
G
H
I
J
K
L
DCL
NBL

Figure 3.6. Analysis of general RPC markers at E16.5. (A-D) *Vsx2* expression remained absent in *Lhx2* CKO retinas (B), and while the expression level did not change in *Rbpj* CKO retinas (D), expression was absent from the apical side of the retina (inset, dashed bracket), indicating the accumulation of postmitotic cells and suggesting premature or excess neurogenesis. (E-H) *Pax6* expression, in RPCs, did not change across control (E, G), *Lhx2* CKO (F), and *Rbpj* CKO (H) retinas. (I-L) *Hes1* expression was decreased in both *Lhx2* CKO (J) and *Rbpj* CKO (L) retinas, with the latter again showing an apical gap of expression (inset, dashed bracket). (M-P) *Hes5* expression levels were decreased in *Lhx2* CKO retinas (N) yet maintained in *Rbpj* CKO retinas (P). In both cases, expression was absent from the apical side of the retina – again indicative of premature neurogenesis. Scale bars: 100 μm.

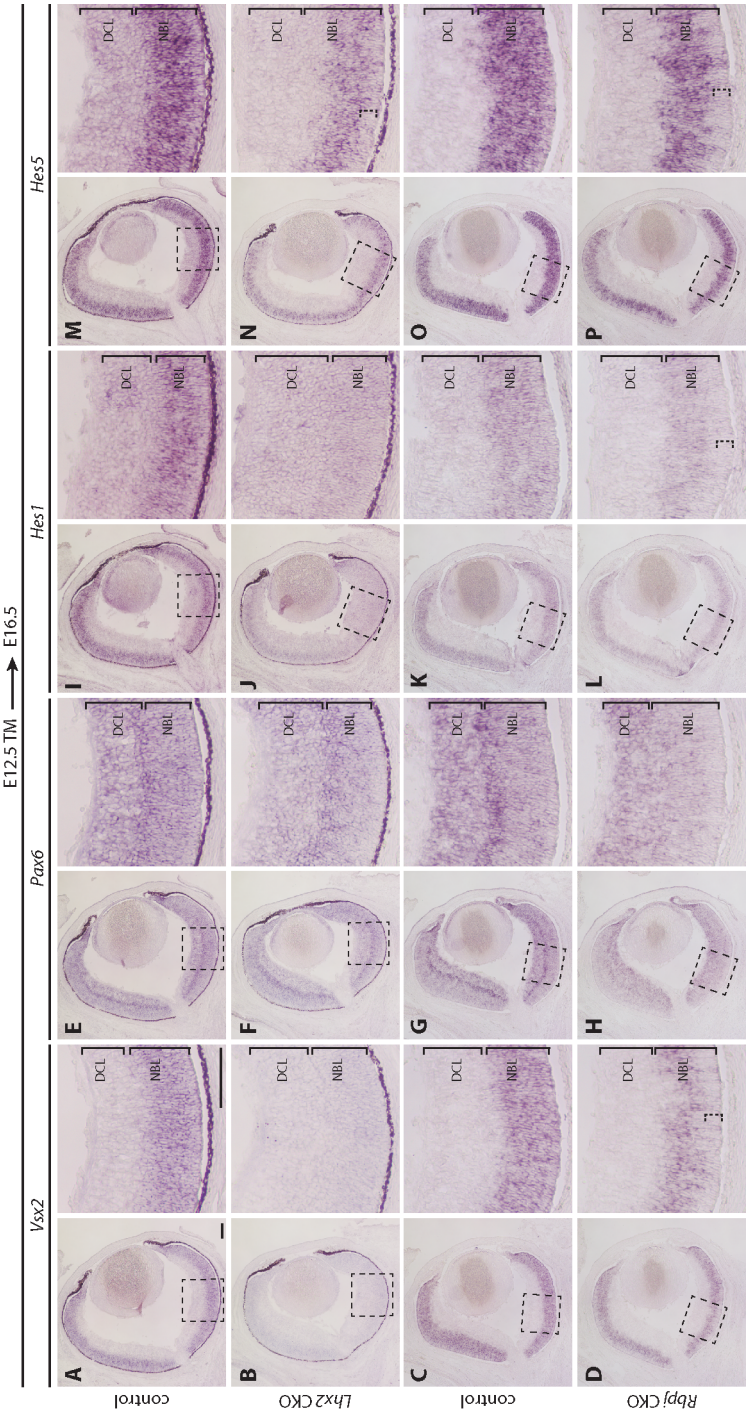

E12.5 TM
E16.5
Vsx2
Pax6
Hes1
Hes5
control
Lhx2 CKO
control
Rbpj CKO
DCL
NBL
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 3.7. Biased RPCs increase after the loss of *Rbpj*, yet this accumulation is prevented with the additional removal of *Lhx2*. (A, B) Ngn2 (A) and Dll1 (B) were both expressed by a subset of RPCs in control retinas. (C, D) Relative to the approximate size of the RPC population, and the thickness of the NBL, *Ngn2* (C) and *Dll1* (D) expression was maintained in *Lhx2* CKO retinas. (E-H) *Ngn2* and *Dll1* increased in *Rbpj* CKO retinas (E, F) and decreased in DCKO retinas (G, H), again relative to the approximate size of the RPC population. NBL, neuroblast layer; DCL, differentiated cell layer. Scale bars: 100 μm.

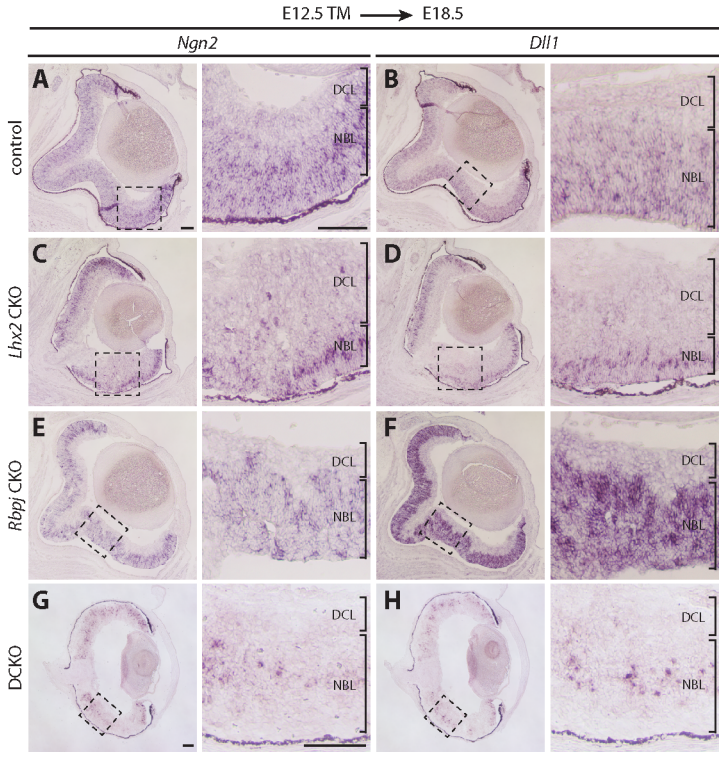
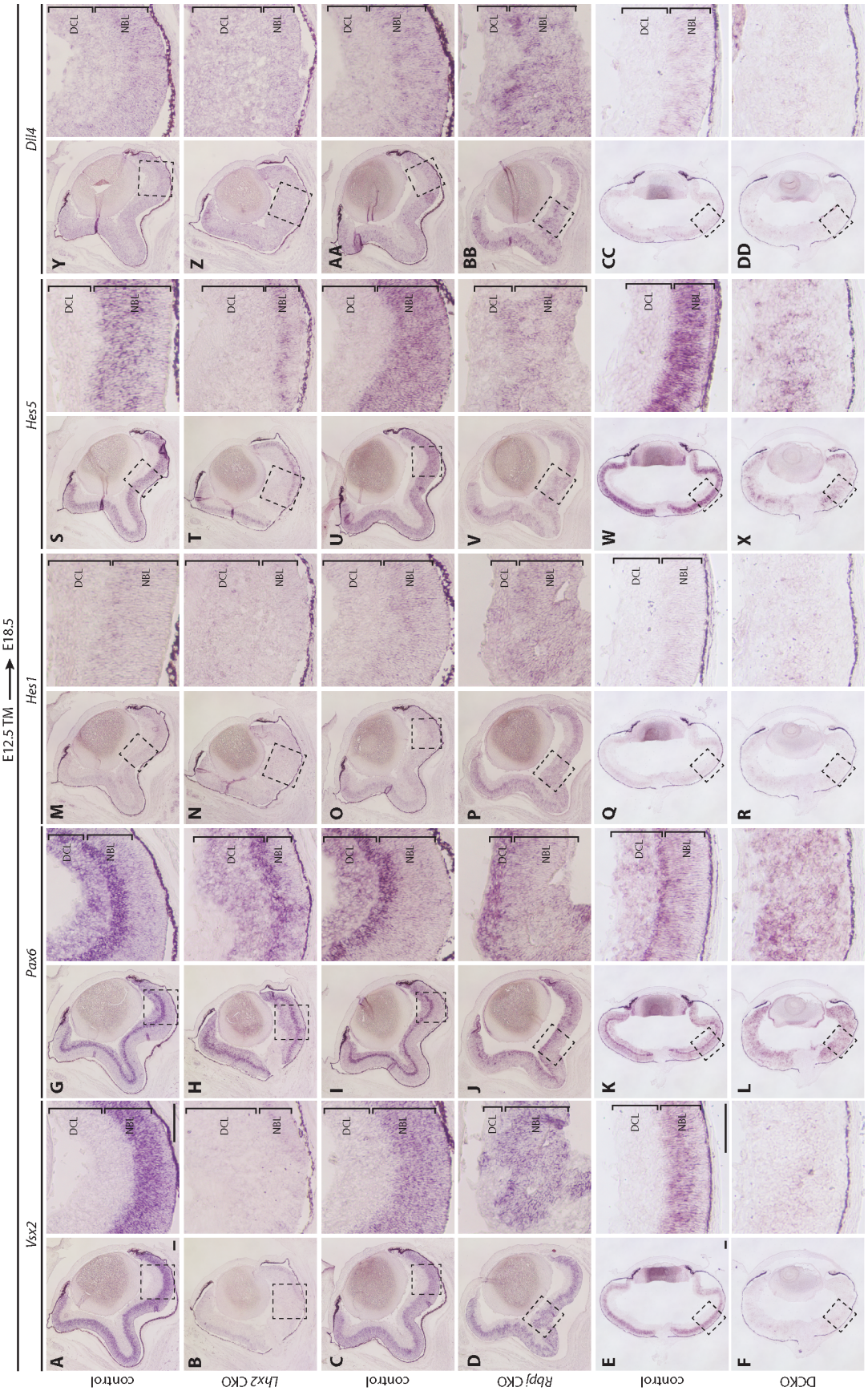

E12.5 TM
E18.5
Ngn2
Dll1
control
Lhx2 CKO
Rbpj CKO
DCKO
A
B
C
D
E
F
G
H
DCL
NBL

Figure 3.8. Analysis of biased and general RPC markers at E18.5. (A-F) Consistent with earlier ages, *Vsx2* expression was absent from *Lhx2* CKO retinas (B), maintained in *Rbpj* CKO retinas (D), and again absent from DCKO retinas (F). (G-L) *Pax6* was expressed at high levels in differentiating amacrine cells and low levels throughout the remaining thickness of the retina in control (G, I, K), *Lhx2* CKO (H), and *Rbpj* CKO samples (J). (M-R) *Hes1* expression was hard to detect in control retinas at this stage (M, O, Q), and did not appear to change in *Lhx2* CKO (N), *Rbpj* CKO (P), or DCKO retinas (R). (S-X) *Hes5* expression decreased in *Lhx2* CKO (T), *Rbpj* CKO (V), and DCKO retinas (X). (Q-T) Biased RPC marker *Dll4*, excluded from Fig. 2 due to space constraints, was difficult to detect in control retinas at this stage, similar to *Hes1* (Y, AA, CC). Expression did not change in *Lhx2* CKO retinas (Z), was increased in *Rbpj* CKO retinas (BB, consistent with the upregulation of *Ngn2* and *Dll1* in Fig. 2), and decreased in DCKO retinas (DD). Scale bars: 100 μm.


E12.5 TM
E18.5
Vsx2
Pax6
Hes1
Hes5
Dll4
control
Lhx2 CKO
control
Rbpj CKO
control
DCKO
DCL
NBL
A
B
C
D
E
F
G
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AA
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CC
DD

Figure 3.9. Both *Lhx2* and *Rbpj* have roles at the level of fate choice. (A-C) Disrupted lamination and overproduction of Pou4f+ RGC precursors were evident in *Rbpj* CKO retinas (B) and exacerbated in DCKO retinas (C). (D-F) Production of Otx2+ photoreceptor precursors was enhanced in *Rbpj* CKO retinas (E), yet normal in DCKO retinas (F). (G-L) Bhlhb5+ and Sox2+ amacrine cell precursors decreased in *Rbpj* CKO (H, K) and DCKO (I, L) retinas, with only a few marker-positive cells remaining (arrows, insets). (M-O) Ptf1a+ amacrine and horizontal precursors, specified in the NBL (M), were lost in *Rbpj* CKO retinas (N) and decreased in DCKO retinas (O). (P) Quantification of cell type production relative to controls and expressed as fold change. The asterisk indicates *Lhx2* CKO data (Gordon et al. 2013) and the pound sign indicates a infinite negative fold change, as zero Ptf1a+ cells were observed in the *Rbpj* CKO counts. Scale bars: *A*, 100 μm; *H, K* (insets), 10 μm.

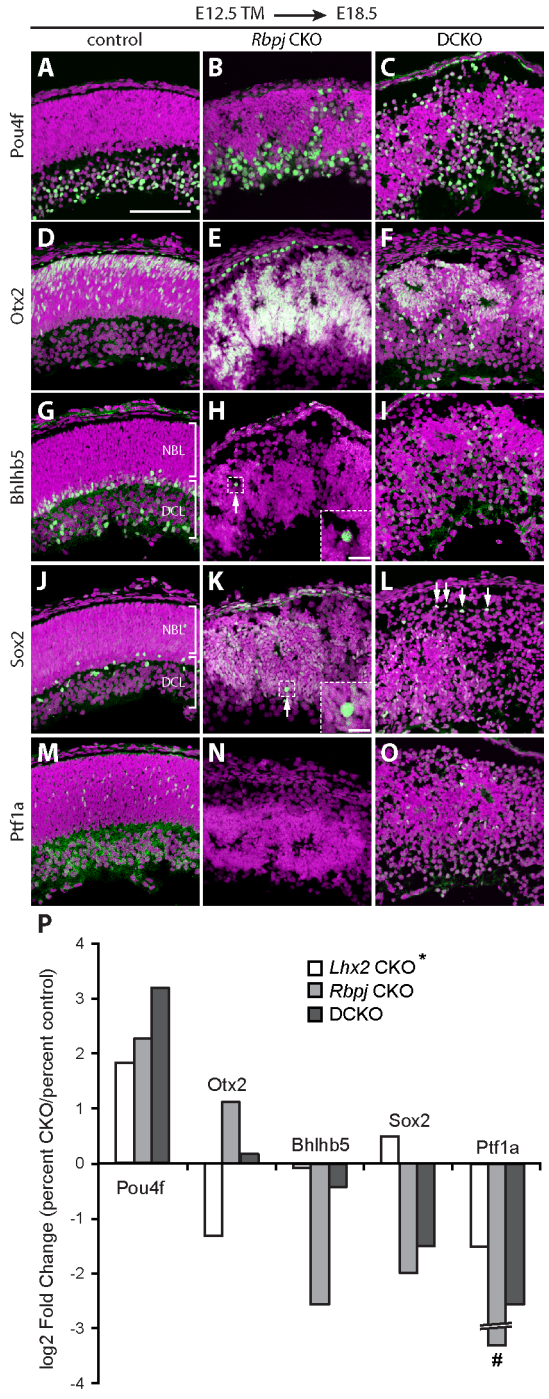

E12.5 TM → E18.5
control
Rbpj CKO
DCKO
Pou4f
Otx2
Bhlhb5
Sox2
Ptf1a
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
NBL
DCL
P
Lhx2 CKO*
Rbpj CKO
DCKO
log2 Fold Change (percent CKO/percent control)
Pou4f
Otx2
Bhlhb5
Sox2
Ptf1a
#

Figure 3.10. Quantification of neurogenic output in the *Rbpj* CKO and DCKO retinas. (A-E) Precursor markers were expressed similarly in controls from both DCKO crosses (Fig. 3) and *Rbpj* CKO crosses (here). (F-K) Rxrγ, Nr2e3, and Thrβ2 mark RGC and cone precursors, rod precursors, and cone precursors, respectively. Expression of Rxrγ (G) and Thrβ2 (K) increased in *Rbpj* CKO retinas relative to controls (F, J), while expression of Nr2e3 (I) remained constant. (L) Quantification confirmed that Otx2+ and Rxrγ+ cells were significantly overproduced in *Rbpj* CKO retinas. Pou4f+ cells increased, though this was not statistically significant. The number of inhibitory interneuron (horizontal and amacrine cell) precursors decreased, though only Bhlhb5 showed a statistically significant change. (M) Pou4f+ cells were significantly overproduced in DCKO retinas, while Otx2 production returned to normal levels. Bhlhb5+, Ptf1a+, and Sox2+ cells all showed a decrease, though the change in Sox2+ was the only statistically significant one. Scale bar: 100 μm.

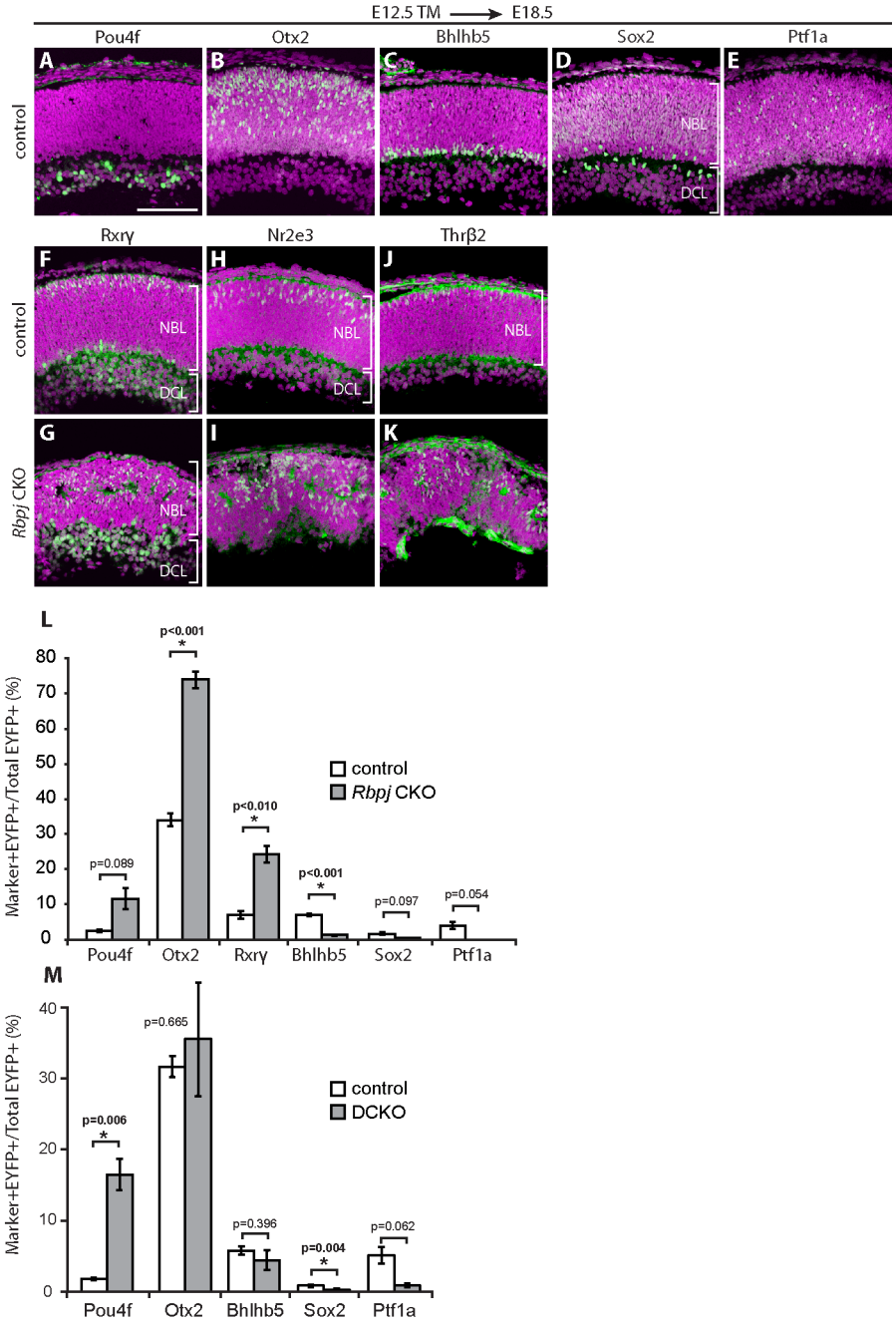
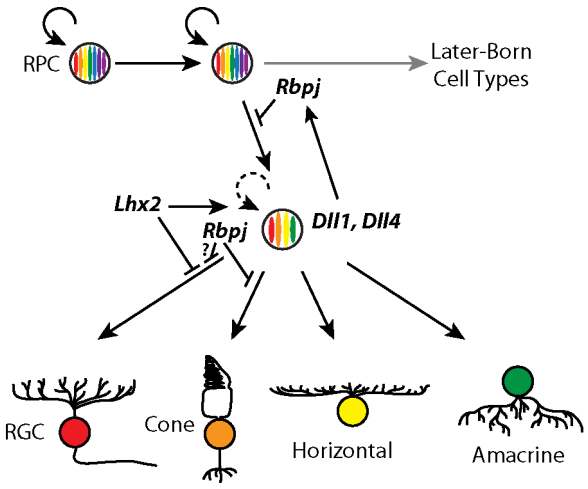

E12.5 TM ⟶ E18.5
Pou4f
Otx2
Bhlhb5
Sox2
Ptf1a
A
B
C
D
E
control
NBL
DCL
Rxrγ
Nr2e3
Thrβ2
F
H
J
G
I
K
Rbpj CKO
L
Marker+EYFP+/Total EYFP+ (%)
control
Rbpj CKO
p=0.089
p<0.001
p<0.010
p<0.001
p=0.097
p=0.054
Pou4f
Otx2
Rxrγ
Bhlhb5
Sox2
Ptf1a
M
control
DCKO
p=0.006
p=0.665
p=0.396
p=0.004
p=0.062

Figure 3.11. Model depicting successive roles in RPC maintenance and fate choice for both *Lhx2* and *Rbpj*. As RPCs exit the cell cycle, proneural transcription factors and Notch ligands are expressed and *Lhx2* is required to drive proliferation for the remaining few cycles (dashed arrow). *Rbpj* facilitates the response of surrounding RPCs to these Notch ligands, temporarily delaying the commitment to differentiation and/or the further generation of biased RPCs. Prior to exit, *Lhx2* and *Rbpj* normally limit the generation of differentially biased RPCs (favoring RGC and photoreceptor production, respectively), though the mechanisms for this remain unclear.


RPC
Later-Born
Cell Types
Rbpj
Lhx2
Rbpj
Dll1, Dll4
RGC
Cone
Horizontal
Amacrine

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CHAPTER 4

LHX2 REGULATES COMPETENCE PROGRESSION IN THE RETINA THROUGH SONIC HEDGEHOG SIGNALING

Abstract

During development, retinal progenitor cells (RPCs) give rise to all seven major retinal cell types in a distinct yet overlapping order, reflected in their own corresponding progression through a series of intrinsically-defined competence states. It remains unknown, however, what intrinsic and/or extrinsic factors regulate the timing of these competence transitions. Previously, we demonstrated that production of retinal ganglion cells (RGCs) is extended in the retina of *Lhx2* conditional knock-out (CKO) mice, revealing a defect in competence progression. RGC production is normally limited through the use of Sonic Hedgehog (SHH) as a negative feedback signal, and here, we show that Shh pathway activity is disrupted in the *Lhx2* CKO retina. This suggests that SHH may limit RGC production by normally promoting a transition in competence, and to this end, we show that SHH pathway activation is sufficient to promote the expression of *Ascl1*, a gene associated with later stages of competence. Further, we show that *Lhx2* is necessary to initiate, yet not maintain, a SHH response, and that conditional inactivation of *Lhx2* with $Ascl1^{CreERT2}$ has no discernible effect – both results consistent with our hypothesis that *Ascl1* marks competence-progressed RPCs that have already responded to RGC-secreted SHH. Together, these results suggest a model in which extracellular signals are capable of modulating the timing of competence progression. Such a role may serve to coordinate the process of retinal neurogenesis, and has implications for the similar time-dependent production of cell types elsewhere in the developing nervous system.

Introduction

In *Drosophila*, neuroblasts of both the ventral nerve cord (Isshiki et al., 2001) and medulla (Li et al., 2013) generate a defined set of progeny over time by sequentially expressing different transcription factors. As these neuroblasts divide asymmetrically, they give rise to ganglion mother cells (GMCs), which inherit and stably express the factor(s) present at their birth. Similarly, an additional cascade of transcription factors is utilized in intermediate neural progenitors (INPs) of the central complex (Bayraktar and Doe, 2013), providing the possibility for a second level of temporal patterning, on top of that presumably inherited from the neuroblast. While many of these transcription factors display cross-regulatory interactions, their expression is not always required (Isshiki et al., 2001; Brzezinski et al., 2011) or sufficient (Li et al., 2013) to ensure progression through the sequence, indicating that other inputs also regulate this process.

A similar, temporally based production of cell types occurs in the vertebrate retina, where all seven major retinal cell types arise in a predictable order from a single pool of multipotent RPCs. The long-standing competence model suggests that each RPC transitions through a series of intrinsically defined competence states (Cepko et al., 1996), but several open questions remain regarding this model. Foremost, the intrinsic factor(s) that define these states are still largely unidentified. One notable exception is *Ikaros*, the mouse ortholog of *Drosophila hunchback*, which promotes the generation of early-born cell types (Elliott et al., 2008). Examination of the *Dicer* CKO retina has identified other candidate factors associated with different competence states (*Prtg*, *Lin28b*, *Ascl1*, and *Sox9*) (Georgi and Reh, 2010; La Torre et al., 2013), yet rather than simply identifying these factors, *Dicer* CKO studies are primarily concerned with how

their expression is regulated; in other words, *how* competence changes are executed. Apart from both lines of investigation, another issue has received relatively little attention: the question of how RPCs know *when* to transition in competence.

Heterochronic culture and transplant experiments first demonstrated that competence was an intrinsic property (Watanabe and Raff, 1990; Morrow et al., 1998; Belliveau et al., 2000; Rapaport et al., 2001), and subsequent studies suggested that RPCs may initiate neurogenesis (Kay et al., 2005) and proceed through the generation of different cell types (Cayouette et al., 2003) on their own intrinsic schedule. Importantly, though, this does not preclude the influence of extrinsic factors. The production of several different retinal cell types is thought to be regulated through feedback (Reh and Tully, 1986; Waid and McLoon, 1998; Belliveau and Cepko, 1999; Zhang and Yang, 2001; Wang et al., 2005), an important mechanism for coordinating lineage progression and ensuring reproducibility in final-state systems (Lander et al., 2009). As fate decisions appear to be made stochastically in the retina (Gomes et al., 2010; He et al., 2012), feedback cues represent excellent candidates controlling variability, through not only affecting binary fate decisions, but potentially modulating the schedule of competence progression. Such a role that has been previously proposed for the RGC-secreted negative feedback signal GDF11 (Kim et al., 2005), and here, we suggest a similar role for SHH by demonstrating that defects in competence progression previously observed in the *Lhx2* CKO retina (Gordon et al., 2013) are likely due to a deficit in SHH signaling activity.

Results

Loss of SHH activity in the *Lhx2* CKO retina

Work in the developing chick retina demonstrated that RGC production is normally limited through the influence of negative-feedback signals derived from newly-born or differentiating RGCs (Waid and McLoon, 1998). Subsequent studies identified SHH and GDF11 as two such factors, showing that manipulations of pathway activity resulted in the expected changes to RGC production (Zhang and Yang, 2001; Kim et al., 2005; Wang et al., 2005). Conditional inactivation of *Lhx2* in early retinal progenitors results in a selective overproduction of RGCs that occurs at the expense of other early-born cell types (Gordon et al., 2013), and thus, a requirement for *Lhx2* in mediating RGC negative-feedback represented an attractive potential mechanism. We chose to focus on SHH, given that its role is well-established and supported by multiple studies (Zhang and Yang, 2001; Wang et al., 2005; Sakagami et al., 2009; Cwinn et al., 2011). To maintain consistency with our previous work (Gordon et al., 2013), we used the inducible *Hes1*^{CreERT2} allele (Yun et al., 2009; Kopinke et al., 2011) to drive conditional inactivation of an *Lhx2* conditional allele (Mangale et al., 2008). We also used the *Ai9* *Rosa*^{tdTomato} allele (Madisen et al., 2010) as a reporter of recombination. Previously, we established a temporal window for *Lhx2* in the regulation of RGC fate, reflected in the asymmetric phenotype generated after inactivation at E12.5 (Gordon et al., 2013). Here, we began by performing inactivation at E11.5, in order to avoid the complication of regional variation in phenotype; subsequent analysis was performed at E15.5, to allow adequate time for recombination. *Shh* is normally expressed by newly-born RGCs that accumulate in the differentiated cell layer (DCL) of the inner retina as neurogenesis

proceeds, and *in situ* hybridization (ISH) of both control (Fig. 4.1A) and *Lhx2* CKO (Fig. 4.1B) eyes at E15.5 revealed similar and expected expression patterns, suggesting that *Lhx2* is not required for the normal expression of *Shh*. This signal is normally received by RPCs in the overlying neuroblast layer (NBL) and detectable via the expression of several target genes, including *Gli1* (Sigulinsky et al., 2008). Assessment of *Gli1* expression at E15.5 revealed the expected pattern in control eyes (Fig. 4.1C), yet a striking and near-complete loss of *Gli1* expression in *Lhx2* CKO eyes (Fig. 4.1D), confirmed by quantitative real-time PCR (qPCR) and western blot (Fig. 4.1E, F). This supported our hypothesis that a loss of Shh signaling in the *Lhx2* CKO retina was responsible for the previously observed RGC overproduction (Gordon et al., 2013). If this hypothesis were correct, the loss of *Gli1* expression would be expected to track directly with RGC overproduction. As inactivation of *Lhx2* at E12.5 generates an asymmetry in RGC production, it provides an opportunity to test this prediction. Accordingly, moving the times of inactivation and analysis to E12.5 and E16.5, respectively, showed no effect on *Shh* expression (Fig. 4.2A, B), yet an asymmetric loss of *Gli1* expression that was specific to the ventral retina and in keeping with our hypothesis (Fig. 4.2C, D). Together, these results suggest that *Lhx2* is required in RPCs to ensure their proper response to RGC-secreted SHH. In the absence of such a response, RGC production continues presumably unchecked.

SHH pathway activation promotes *Ascl1* expression

During the early stages of neurogenesis, RPCs are capable of generating four different cell types: RGCs, horizontal cells, cones, and amacrine cells. SHH acts as a negative feedback signal to limit RGC production, and thus, manipulations of pathway

activity that produce either an increase or decrease in RGC number result in reciprocal changes to the production of these other early-born cell types (Wang et al., 2005; Cwinn et al., 2011). However, the loss of both LHX2 (Gordon et al., 2013) and SHH (Wang et al., 2005) also extends RGC production. Thus, SHH may not only limit RGC production in favor of other early-born fates, but promote a transition in competence that favors the production of later-born fates. *Ascl1* is a proneural bHLH transcription factor whose expression begins in the central retina at approximately E12.5 and marks a lineage-restricted subset of RPCs that do not give rise to RGCs (Brzezinski et al., 2011). As a result, it has been used as a marker of competence progression (Georgi and Reh, 2010; La Torre et al., 2013). In contrast, previous work implicating LHX2 and SHH in competence progression has relied on RGC birthdating (Wang et al., 2005; Gordon et al., 2013), and we therefore wished to address whether the expression of *Ascl1*, as a molecular marker of competence progression, was also affected. Inactivation of *Lhx2* at E12.5 led to a symmetric and near-complete loss of LHX2 (Fig. 4.3A, B), yet a decrease in ASCL1 that appeared specific to the ventral retina (Fig. 4.3C, D), in line with the similar loss of *Gli1* expression detailed above. To quantify this effect, we performed qPCR on control and *Lhx2* CKO samples from E11.5-E15.5 and confirmed a significant reduction in *Ascl1* expression (Fig. 4.3E). This implies that SHH signaling, lost in the *Lhx2* CKO retina, may normally promote ASCL1 expression, in line with recent results reporting a similar regulatory relationship in P19 embryonal carcinoma cells (Voronova et al., 2011). To test this hypothesis, we first cultured E12.5 wild-type retinal explants for 24 hours in the presence of purmorphamine (Pur), a small-molecule activator of SHH signaling (Wu et al., 2002; Sinha and Chen, 2006). We have shown previously that *Gli1* expression is

quickly lost in retinal explants, presumably due to selective RGC death (Sigulinsky et al., 2008). Predictably, therefore, vehicle-treated explants did not display significant *Gli1* expression after 24 hours (Fig. 4.3F). However, treatment with Pur resulted in the rescue and/or maintenance of *Gli1* expresssion (Fig. 4.3G), providing a clear difference in pathway activity between the two conditions. This resulted in an increase in both the number of $ASCL1^{+}$ cells, as well as the apparent level of ASCL1 expression, with much stronger staining evident in the Pur-treated explant (Fig. 4.3H-J). Together with the loss of both *Ascl1* and *Gli1* observed in *Lhx2* CKO retina, this result suggests that SHH may normally promote competence progression through the regulation of *Ascl1*.

SHH pathway activation results in partial rescue of the *Lhx2* CKO phenotype

While SHH pathway activation is capable of promoting *Ascl1* expression in wild-type retinal explants, we next wished to address whether a similar approach could rescue different aspects of the *Lhx2* CKO phenotype. Previous experiments demonstrated that SHH activity was lost as soon as four days after inactivation (Fig. 4.1), and thus here we used a similar paradigm for our experiments. *Lhx2* was inactivated at E11.5, and at E15.5 retinas were removed and cultured as explants in the presence or absence of Pur. To assess the effect on RGC production, explants were cultured for 24 hours before being exposed to EdU for 2 hours, to label actively dividing RPCs. Subsequently, explants cultured for an additional 24 hours, to allow labeled progenitors to progress through the cell-cycle and give rise to postmitotic precursors. This is similar to previous birthdating experiments (Wang et al., 2005; Gordon et al., 2013), and allowed us to generate a discrepancy in SHH activity before assessing RGC production with the cell-type specific

marker *Pou4f* (also known as *Brn3*). After processing and analysis, *Vsx2*, strongly expressed in control retinas (Fig. 4.4A, B) was lost *Lhx2* CKO explants (Fig. 4.4C, D), confirming a high level of recombination and was consistent with our previous findings (Yun et al., 2009; Gordon et al., 2013). Similar to what was observed with wild-type E12.5 explants, there was a clear difference in signaling activity between vehicle- and Pur-treated explants of both genotypes, as assessed with *Gli1* transcription (Fig. 4.4E-H). However, while *Lhx2* CKO explants displayed the expected increase in RGC production, Pur treatment did not affect the rate of RGC production in either control or *Lhx2* CKO explants (Fig. 4.4I-M). This was unexpected, and suggests that SHH activity may not be sufficient to affect RGC production at later stages (E15.5+).

To determine whether Pur treatment could rescue *Ascl1* expression, we stained both control and *Lhx2* CKO explants for ASCL1. Similar results were obtained for expression patterns for *Vsx2* and *Gli1* (Fig. 4.5A-H), and in control explants, Pur treatment appeared to modestly increase the expression of ASCL1 over that seen with vehicle (Fig. 4.5I, J), though this was difficult to discern given the already high level of expression at this stage. In *Lhx2* CKO explants there was a clear decrease of ASCL1 expression in vehicle-treated explants (Fig. 4.5K) and a corresponding increase in Pur-treated explants (Fig. 4.5L). SOX9, another late-stage RPC marker associated with competence progression (Georgi and Reh, 2010; La Torre et al., 2013), did not appear to change with treatment in the control explants (Fig. 4.5M, N). In contrast, vehicle-treated *Lhx2* CKO explants displayed a decrease in Sox9 expression (Fig. 4.5O) that was partially rescued with Pur treatment (Fig. 4.5P). These results suggests that a deficit in

SHH signaling likely explains the failed competence progression of RPCs observed in the *Lhx2* CKO retina.

Given that these roles of *Ascl1* and *Sox9* were characterized in *Dicer* CKO retinas that similarly fail to progress in competence due to a lack of several critical micro-RNAs (miRNAs), we wondered whether the expression of these miRNAs was also affected in the *Lhx2* CKO retina. However, using identical primers to assess their relative levels via qPCR, we were unable to show any change in expression for miR-9, miR-183, let-7a, or let-7f (Fig. 4.5Q). This indicates that the potential roles of both *Lhx2* and *Shh* in promoting competence progression may exist in parallel with or downstream of *Dicer*.

Inactivation of *Lhx2* with *Ascl1*^{CreERT2} yields no obvious phenotype

Previously, we showed that the inactivation of *Lhx2* is only capable of affecting RGC production within a limited window, as inactivation at E10.5, yet not E13.5, resulted in the selective overproduction of RGCs (Gordon et al., 2013). Further, this window appeared to close first in the dorsal retina, as inactivation of *Lhx2* at E12.5 resulted in an asymmetric overproduction of RGCs that was confined to the ventral retina, despite symmetric loss of LHX2 (Gordon et al., 2013). Here, we show that inactivation of *Lhx2* has a similar effect on the SHH pathway readout *Gli1*, with inactivation at E11.5 and E12.5 resulting in a similar symmetric and asymmetric loss of expression, respectively. This is most likely explained by a similar, yet reciprocal, anatomical bias – as the initiation of neurogenesis begins in the dorsal-central retina before spreading both ventrally and peripherally as a wave (Hufnagel et al., 2010). It is feasible that this bias in the spread of the neurogenic wave creates a similar bias in the production and response to RGC-secreted SHH; therefore, *Lhx2* appears to preclude a

SHH response only in RPCs that have not already been exposed to SHH. *Ascl1* expression also follows slightly behind the neurogenic wave and is first detectable at E12.5 in the same dorsal-central region (Hufnagel et al., 2010), coinciding both spatially and temporally with the presumed onset of SHH signaling. Here, we show that the SHH pathway agonist Pur is capable of promoting *Ascl1* expression (Fig. 4.3), and therefore hypothesized that *Ascl1* expression marks competence-progressed RPCs which are no longer capable of generating RGCs due to the receipt of an RGC-derived SHH negative feedback signal. Given that *Lhx2* is not necessary to maintain, but only initiate, this response, we predicted that ASCL1$^+$ RPCs would be unaffected by the loss of *Lhx2*. To test this, we used *Ascl1*^{CreERT2} (Kim et al., 2011) to conditionally inactivate *Lhx2* beginning at E12.5. Administration of a single dose of tamoxifen at either E11.5 or E12.5 resulted in very low levels of recombination, yielding approximately 1-10 tdTomato+ cells per retinal section (data not shown). Thus, similar to others (Brzezinski et al., 2011), we administered tamoxifen at both E12.5 and E13.5 in order to increase the total number of ASCL1$^+$ cells labeled. ASCL1$^+$ RPCs primarily generate small clones that result from only one or two divisions, though many do continue to proliferate until postnatal ages and contribute to the generation of later-born cell types (Brzezinski et al., 2011). Therefore to increase our likelihood of observing an effect, yet at the same time avoid complications with delivery that result from tamoxifen treatment, we analyzed these mice at E18.5. In addition, this age was consistent with our previous experiments utilizing *Hes1*^{CreERT2} (Gordon et al., 2013). In line with our prediction, however, we were unable to identify any differences in the number or distribution of these cells in control and *Lhx2* CKO eyes (Fig. 4.6A, B). While a small proportion (~1%) of tdTOMATO$^+$ cells did assume the

RGC fate (Fig. 4.6A', B'), in contrast with what has been previously reported (Brzezinski et al., 2011), there was no obvious difference in their number between genotypes. To ensure that *Lhx2* had been properly deleted, we examined several retinas at E15.5, as our previous work demonstrated that only 48 hours was needed for recombination and near-complete loss (Gordon et al., 2013). LHX2 expression normally marks most, if not all, RPCs and is quickly lost in postmitotic cells; accordingly, while tdTOMATO$^+$ cells located outside of the NBL would not normally be expected to express LHX2, many of those observed within the NBL were in fact negative for LHX2 (Fig. 4.6C), confirming that ASCL1$^+$ cells which continue as RPCs lose their expression of LHX2. Subsequent quantifications confirmed that there were no effects on proliferation, as the total number of tdTOMATO$^+$ cells and the percentage that remained proliferative (as assessed by co-expression of PCNA), were unchanged at E18.5 (Fig. 4.6D, E). Similar confirmation was obtained for the lack of effect on RGC production (Fig. 4.6F). These results contrast dramatically with the loss of RPCs and corresponding overproduction of RGCs that is observed after deletion using *Hes1*^{CreERT2} at the same time point (Gordon et al., 2013). This supports our model (Fig. 4.7), in which *Lhx2* limits RGC production through its requirement in initiating a SHH response, and *Ascl1* marks RPCs that have already been exposed to SHH.

Discussion

Previously, we demonstrated that conditional inactivation of *Lhx2* during early stages of retinal neurogenesis leads to the selective and sustained production of RGCs (Gordon et al., 2013). In the current study, we aimed to identify the mechanism responsible for this phenotype, and focused on a potential role for *Lhx2* in mediating

SHH signaling activity. We found that the expression of *Gli1*, a transcriptional readout of the pathway, was lost in both spatial and temporal correlation with the previously observed RGC overproduction. *Ascl1* expression was decreased in the *Lhx2* CKO retina, consistent with a failure in competence progression, and treatment of retinal explants with the SHH pathway agonist Pur increased *Ascl1* expression in both *Lhx2* CKO and wild-type retinal explants. Finally, consistent with our prediction, inactivation of *Lhx2* in the *Ascl1* lineage did not appear to have any significant effect on proliferation or cell fate. Together, these results suggest that RGC-secreted SHH not only serves as a negative-feedback signal to limit the further production of RGCs, but promotes a transition in competence in part through regulation of *Ascl1*.

The model depicted in Fig. 4.7 summarizes these results, supporting the SHH-mediated regulation of competence. Newly specified RGC precursors migrate to the basal side of the retina and accumulate to form the developing ganglion cell layer, where *Shh* is clearly expressed (Jensen and Wallace, 1997; Levine et al., 1997; Neumann and Nuesslein-Volhard, 2000; Sigulinsky et al., 2008). SHH drives pathway activity in RPCs of the overlying NBL (Jensen and Wallace, 1997; Sigulinsky et al., 2008) to promote proliferation (Levine et al., 1997; Sigulinsky et al., 2008) and limit the further production of RGCs (Zhang and Yang, 2001; Wang et al., 2005; Sakagami et al., 2009; Cwinn et al., 2011). We propose here that SHH also promotes a competence transition, through the regulation of *Ascl1* expression. *Lhx2*, expressed in most if not all RPCs (Tétreault et al., 2009; Gordon et al., 2013), is required for mediating the response to RGC-secreted SHH (this study), and we hypothesize that upon inactivation with $Hes1^{CreERT2}$, the loss of *Lhx2* results in a corresponding loss of these functions and a scenario in which many RPCs

continue producing ectopic RGCs at the expense of other early-born and later-born cell types.

LHX2-mediated regulation of the SHH pathway

While we demonstrate here that *Lhx2* is clearly required in RPCs to ensure their normal response to SHH, it remains unclear how *Lhx2* specifically affects the pathway. Pur is a well-characterized small molecule acting on the transmembrane protein *Smoothened* (*Smo*) (Sinha and Chen, 2006), and thus the ability of Pur treatment to rescue *Gli1* expression suggests that any defect in the pathway exists at or above the level of SMO. Binding of SHH to its canonical receptor *Patched1* (*Ptc1*) relieves constitutive inhibition of SMO, and thus, interfering with the normal expression of *Ptc1* results in a gain-of-function phenotype (Goodrich et al., 1997). In addition, *Ptc1* is a target of the pathway and, along with *Hedgehog interacting protein* (*Hhip*), provides negative feedback by sequestering Shh and limiting its diffusion. The loss of such negative regulators therefore would not explain the *Lhx2* CKO phenotype. However, several SHH co-receptors exist that positively promote pathway activity in certain contexts (Tenzen et al., 2006; Allen et al., 2007; 2011), forming multimolecular complexes with PTC1 and necessary for signal transduction (Izzi et al., 2011); these include *Cdon* (*CAM-related downregulated by oncogenes*), *Boc* (*brother of Cdon*), and *Gas1* (*growth arrest-specific 1*). However, these co-receptors also negatively regulate pathway activity in other contexts, and, in short, are unlikely to play a functional role in RPCs given their expression patterns (P.G., unpublished observations; Lee and Fan, 2001; Zhang et al., 2009; Fabre et al., 2010; Sánchez-Arrones et al., 2013). It is possible that the loss of *Lhx2* affects some aspect of the processing or presentation of SHH itself, yet we have no

evidence to suggest this, and show that *Shh* is actively transcribed in the absence of *Lhx2* during both late (this paper) and early (Yun et al., 2009) stages of eye development. Given that dorsal RPCs in the E12.5 *Lhx2* CKO display a robust *Gli1* response despite the symmetric loss of *Lhx2* observed in that paradigm, this possibility seems unlikely. In addition to directly regulating these or other pathway components, however, *Lhx2* may also affect pathway activity indirectly. In support of this, previous work in our lab suggested that *Lhx2* may serve to couple signaling pathways with expression of specific target genes (Yun et al., 2009). While likely, then, such an indirect effect could occur through a variety of mechanisms, including the regulation of other trans-acting factors or even the control of chromatin state.

Our results raise another intriguing question about the control of SHH pathway activity, in that *Lhx2* is required to initiate, yet not maintain, *Gli1* expression. One potential explanation for this centers around both modeling and experimental data which show that SHH signaling can operate as a bistable switch, requiring a higher threshold of input to initiate rather than maintain a response (Lai et al., 2004; Balaskas et al., 2012; Panovska-Griffiths et al., 2013). It is feasible that loss of *Lhx2* may compromise, rather than completely preclude, pathway activity, and as a result, the threshold of activity required to initiate a response may never be reached in the *Lhx2* CKO retina. Such a scenario suggests, as mentioned above, that *Lhx2* may regulate pathway activity or response indirectly, rather than through direct regulation of key pathway components.

RGC production and negative feedback

The loss of *Gli1* expression in the *Lhx2* CKO retina implies that a lack of SHH-mediated negative feedback may be responsible for the RGC overproduction observed in

Lhx2 CKO retinas (Gordon et al., 2013). Yet while Pur treatment was capable of restoring *Gli1* expression in *Lhx2* CKO retinal explants, we were surprised that it did not modulate RGC production. In the wild-type retina, *Gli1* is expressed quite strongly in RPCs at E15.5 (Sigulinsky et al., 2008). Thus, wild-type explants had presumably seen and responded to endogenous SHH before their removal and culture, and it is less surprising that treatment with Pur had no effect on their RGC production. Accordingly, all previous studies demonstrating SHH-mediated RGC negative feedback *in vitro* were performed on wild-type tissue isolated during earlier, peak stages of RGC production (approximately E12-E13 in mice) (Zhang and Yang, 2001; Wang et al., 2005). Given that RGC production is extended in the *Lhx2* CKO retina (Gordon et al., 2013), however, a lack of effect on their RGC production was unexpected, and suggests that a limited temporal window may exist in which SHH can act as a negative-feedback signal. This idea is consistent with the observation that many SHH target genes in the retina respond differently to pathway stimulation during early and late stages of neurogenesis (McNeill et al., 2012), and determining which pathway targets are important for the role of SHH in RGC negative feedback will provide clarification. *Ascl1*, for example, has been proposed as a target of SHH signaling (this paper; Voronova et al., 2011), yet while it marks lineage-restricted RPCs unable to generate RGCs (Brzezinski et al., 2011) likely does nothing to limit RGC competence itself (Brzezinski et al., 2011; Hufnagel et al., 2013).

LHX2 and SHH may promote proliferation through regulation of *Ascl1*

Early studies of SHH function in the retina reported differing roles in the promotion of either differentiation (Neumann and Nuesslein-Volhard, 2000; Shkumatava et al., 2004; Shkumatava and Neumann, 2005) or proliferation (Jensen and Wallace,

1997; Levine et al., 1997; Moshiri and Reh, 2004). Subsequently, it has been proposed that this discrepancy may be explained by functions for SHH in accelerating the cell-cycle yet also driving cell-cycle exit (Locker et al., 2006; Agathocleous et al., 2007), which would result in the conversion of slow cycling progenitors into transit-amplifying cells that are close to exit. Gain-of-function experiments in mice support this idea, as an increase in SHH activity produces a significant yet transient increase in proliferation, as well as developmentally premature cell-cycle exit (Cwinn et al., 2011). Previously, we have proposed a similar role for *Lhx2* in supporting the proliferation of biased RPCs, which we defined as close to cell-cycle exit and limited in proliferative capacity (Gordon et al., 2013). Here, we link these separately proposed functions by demonstrating that SHH signaling activity is lost upon deletion of *Lhx2*. Further, we show that SHH plays a role in promoting the normal expression of *Ascl1*. This provides a potential explanation for the proliferative defects observed in both *Lhx2* and *Shh* mutant mice, as multiple lines of evidence now support a role for *Ascl1* in promoting RPC proliferation, despite the fact that it is traditionally associated with a commitment to differentiate. Loss of *Ascl1* modestly decreases progenitor cell number (Brzezinski et al., 2011), while misexpression promotes proliferation and delays cell-cycle exit both autonomously and non-autonomously (Hufnagel et al., 2013). This latter finding is in line with its proposed regulation of both positive cell-cycle regulators (Castro et al., 2011) as well as the Notch ligand *Dll1* (Nelson et al., 2009). *Ascl1* is not expressed in all RPCs, though, and other genes are also likely to be involved in explaining the *Lhx2* CKO phenotype. *Cyclin D1* (*Ccnd1*), for example, is a another target of the SHH pathway (Wang et al., 2005; Locker et al., 2006) similarly lost in *Lhx2* CKO retinas (Gordon et al., 2013) and itself previously

implicated by our lab in promoting the proliferation of RPCs close to cell-cycle exit (Das et al., 2009; 2012).

Conclusion

This study identifies SHH signaling as a potential mechanism explaining the defects previously observed in the *Lhx2* CKO retina; namely, the selective and sustained production of RGCs. Additionally, we propose that *Ascl1* represents a target of SHH signaling in the developing retina. While a role for SHH in RGC negative feedback is already established, our work implicates SHH as an extracellular cue that may also provide feedforward regulation by promoting a normal transition in competence. While perhaps not absolutely required for this transition, RGC-secreted signals such as SHH and GDF11 may serve to dictate timing and thus fine-tune the process of retinal histogenesis. Further studies are required however, to determine 1) how the loss of *Lhx2* affects SHH signaling, 2) which SHH pathway targets are involved in limiting RGC production, and 3) the relation of *Lhx2* and SHH signaling to other mechanisms associated with the promotion of competence transitions.

Materials and methods

Animals

The *Lhx2* conditional allele was generated by Mangale et al., and mice were genotyped as described. The $Hes1^{CreERT2}$ knock-in allele was generated by Kopinke et al., and mice were genotyped as described; for all analyses, both mutant and control animals were heterozygous for $Hes1^{CreERT2}$. The $Ascl1^{CreERT2}$ knock-in allele and the *Ai14 tdTomato* reporter allele were generated by Madisen et al. and obtained from Jackson

Laboratory, with mice were genotyped as described. Embryonic age determinations were based on plug date and morphological criteria. All animal use and care was conducted in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee and set forth in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals. Efforts were made to minimize discomfort to animals and when possible, the number of animals needed per analysis was kept to a minimum.

Administration of tamoxifen and EdU

TM (Sigma T5648) was dissolved in corn oil (Sigma C8267) at a concentration of 20 mg/ml and 0.2 mg TM per gram body weight was administered to pregnant dams by oral gavage at indicated stages with 22G 1.5 inch feeding needle.

Immunohistochemistry and *in situ* hybridization

Embryo heads or eyes were dissected in HBSS or PBS and fixed in 4% PFA for 2 hours on ice. Tissue was washed with PBS, put through a gradient of sucrose solutions, embedded in OCT (Sakura Finetek, Torrance, CA), and stored at -80°C. Frozen tissues were subsequently sectioned on a cryostat at a thickness of 12 μm and stored at -20°C.

Primary antibodies used were: rabbit anti-LHX2 (Edwin Monuki, University of California, Irvine, CA; 1:50), goat anti-GFP (Rockland; 1:5000), rabbit anti-GFP (Abcam, 1:4000), rabbit anti-CCND1 (Lab Vision; 1:400), mouse anti-PCNA (DAKO; 1:500), rabbit anti-pHH3 (Upstate; 1:500), goat anti-BRN3 (Santa Cruz; 1:50), rabbit anti-SOX2 (Abcam; 1:400), anti-RXRγ (Santa Cruz; 1:200), anti-NR2E3 (Anand Swaroop, National Eye Institute, Bethesda, MD; 1:100), goat anti-BHLHB5 (Santa Cruz;

1:1000), guinea pig anti-PTF1A (Jane Johnson, University of Texas Southwestern, Dallas, TX; 1:8,000), and rabbit anti-OTX2 (Chemicon; 1:15,000). Primary antibodies were followed with species-specific secondary antibodies conjugated to Alexa Fluor 488, 568, or 647 (Invitrogen/Molecular Probes, Eugene, OR). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Fluka). 5-ethynyl-2'-deoxyuridine (EdU) was used in culture experiments to label actively dividing cells, and subsequent detection of EdU was performed with the Click-it Cell Reaction Buffer Kit (Invitrogen) and fluorescently labeled azides, following any relevant immunohistochemistry and DAPI treatment. Panels showing fluorescence-based protein detection are single scan confocal images obtained with a Fluoview 1000 confocal microscope (Olympus).

For *in situ* hybridization, tissue dissected in RNase-free 1XPBS and fixed in 4% PFA for 2 hours on ice before going through routine washes (with 1X PBS) and a gradient of sucrose solutions, as with immunohistochemistry. However, all solutions were prepared to be RNase-free. Hybridizations were performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993), and probes used in this study were digoxigenin-labeled anti-sense probes against *Shh* and *Gli1*.

Western Blots

[Randy Ringuette – Valerie Wallace Lab]

Quantitative real-time PCR

Embryos were dissected, and eyes removed, in RNase-free conditions. After removal of all surrounding tissue, retinas were flash frozen using liquid nitrogen and stored at -80°C. Total RNA was isolated with Qiashredder columns (Qiagen) and the

RNeasy Micro Kit (Qiagen) before storage at -20°C. For all genes examined, cDNA was synthesized using the Superscript VILO kit cDNA kit(Invitrogen), and the primers used are listed in Table 4.1. All were designed to span exon-exon junctions when possible; for single-exon genes, such primer design was not possible and thus experiments were compared to RT- controls. Specificity was confirmed using dissociation curves and gel electrophoresis, and similar efficiency of primer sets ensured using serial dilutions of cDNA. *Gapdh* was used as an endogenous control for all samples. For all micro-RNAs examined, experiments were performed as described in (La Torre et al., 2013). Reactions were run on an ABI 7300 Real-Time PCR System using Power SYBR Green Master Mix (Invitrogen). The delta delta Ct method was used for determining changes in expression.

Retinal explant cultures

Dissection and removal of surrounding tissues was performed in HBSS. Retinas were then cultured as explants with the lens and vitreal chamber intact in 24-well culture plates. Culture media (1X DMEM/F12, US Biological) was supplemented with 0.6% glucose, 0.1125% $NaHCO_3$, 5mM Hepes, 1% FBS, Glutamax (0.5X, Invitrogen), Penicillin/Streptomycin (1X, Invitrogen), and N2 supplement (1X, Invitrogen). Explants were incubated at 37° C and 5% CO2 with constant nutation to ensure perfusion of solutions through the entire retina.

To test the effects of Shh pathway activation, one explant per embryo was treated with 1 μM purmorphamine (EMD Chemicals) while the contralateral explant was treated with 0.04% DMSO as a vehicle control. For wild-type explants dissected at E12.5, each explant was placed in a single well of the 24-well culture plate in a total culture volume (media plus treatment) of 1ml. At the end of the 24 hour culture period, tissue was fixed

and processed for immunohistochemistry as described above. For both control and *Lhx2* CKO explants dissected at E15.5, each explant was again placed in a single well of the 24-well culture plate, yet in a total culture volume of 2ml. After 24 hour in culture, 1ml of culture media was removed and EdU added at a concentration of 10 μM. Explants were cultured in this media for 2 hours before being removed and placed back into a culture volume of 2ml (1ml of the original media plus 1ml of new media) for an additional 24 hours. At the end of the culture period, tissue was fixed and processed for immunohistochemistry as described above.

Quantification and statistical analyses

For all quantifications, we used a minimum of three animals per genotype, across two or more separate litters. Cell counts and area measurements were done using Photoshop CS6 and ImageJ software. For determining significance in all comparisons we used an alpha level of 0.05 and a two-sided Aspin-Welch-Satterthawaite Student's t test, which assumes normal distribution but unequal variance. These calculations were performed using JMP Pro 11.0 statistical software. All graphed values are represented as the mean ± standard error.

Table 4.1 Primer sequences for qPCR

Gene	Sequence	Tm	Product size
Gapdh	5’ -ctggagaaacctgccaagtatg- 3’	59	205bp
	5’ -cattgtcataccaggaaatgagc- 3’	59	
Lhx2	5’ -tctgaccgctactacctgctg- 3’	59	156bp
	5’ -cacagagaaccgcctgtagtag- 3’	58	
Gli1	5’ -gggaacagaaggactttctgg- 3’	59	116bp
	5’ -ggaaagagagatccttcagtgc- 3’	58	
Ascl1	5’ -cccaactactccaacgacttg- 3’	58	107bp
	5’ -cagcagctcttgttcctctg- 3’	57	

Figure 4.1 *Gli1* expression is lost after *Lhx2* CKO. (A) In control retinas, *Shh* expression in newly-specified RGCs is evident within the DCL (arrowheads). (B) In Lhx2 CKO retinas, Shh expression is maintained. (C) RPCs residing in the overlying NBL normally express *Gli1* in response to RGC-secreted Shh. (D) Loss of *Lhx2* results in the near-complete loss of *Gli1* expression in RPCs. (E) Confirmation and quantification of *Lhx2* and *Gli1* loss in the *Lhx2* CKO retina via quantitative PCR. (F) Gli1 protein expression, clearly present in both P0 wild-type and E15.5 control retinas is completely lost after *Lhx2* CKO; γ-tubulin is used as a loading control. Boxed regions are shown as high magnification panels immediately to the right of all panels. Abbreviations: D, dorsal; V, ventral; DCL, differentiated cell layer; NBL, neuroblast layer. Scale bar, 100 μm.

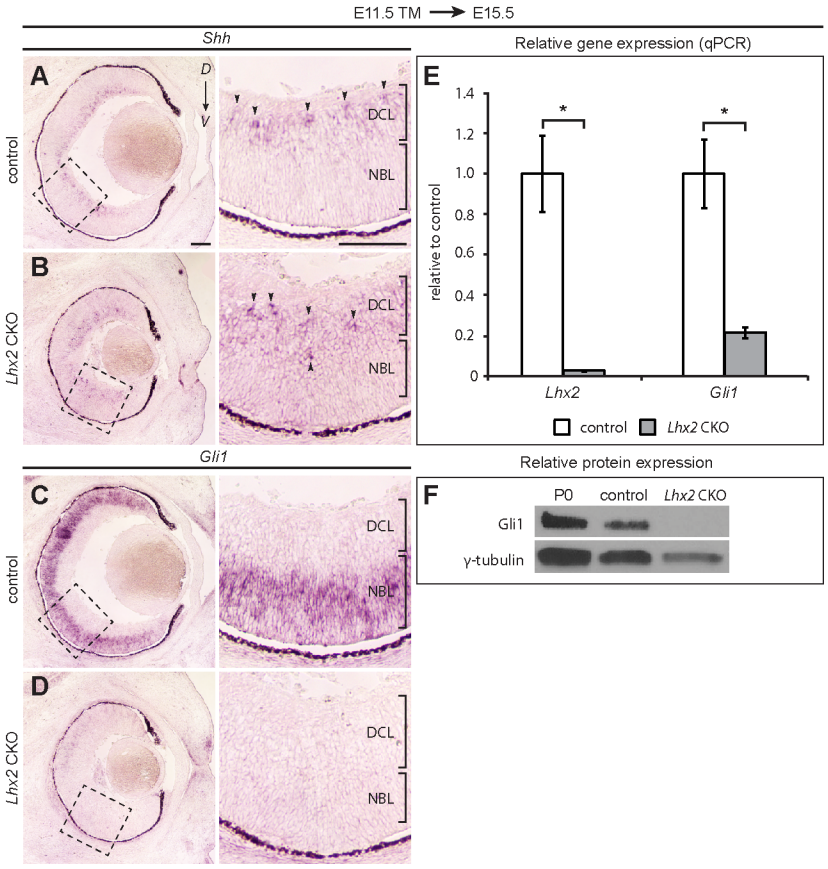

E11.5 TM → E15.5
Shh
A
B
control
Lhx2 CKO
D
V
DCL
NBL
Gli1
C
D
Relative gene expression (qPCR)
E
relative to control
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
Lhx2
Gli1
control
Lhx2 CKO
Relative protein expression
F
P0
control
Lhx2 CKO
Gli1
γ-tubulin

Figure 4.2 E12.5 *Lhx2* CKO results in asymmetric loss of *Gli1* expression. (A) *Shh* is expressed by newly-specified RGCs located within DCL, though at very low levels. (B) *Shh* expression is still evident, though again at low levels, in the DCL of *Lhx2* CKO retinas, which displays an increase in thickness at this age due to RGC overproduction. (C) *Gli1* is expressed at comparable levels in RPCs found on both the dorsal and ventral side of control retinas. (D) *Gli1* expression is maintained in the dorsal region of *Lhx2* CKO retinas, though lost ventrally. In addition, the apical gap in expression (bracket) indicates a thinning NBL and depletion of RPCs. Boxed regions are shown as high magnification panels, with dorsal regions immediately to the right of all panels and ventral regions immediately right of those.Scale bars, 100 μm.

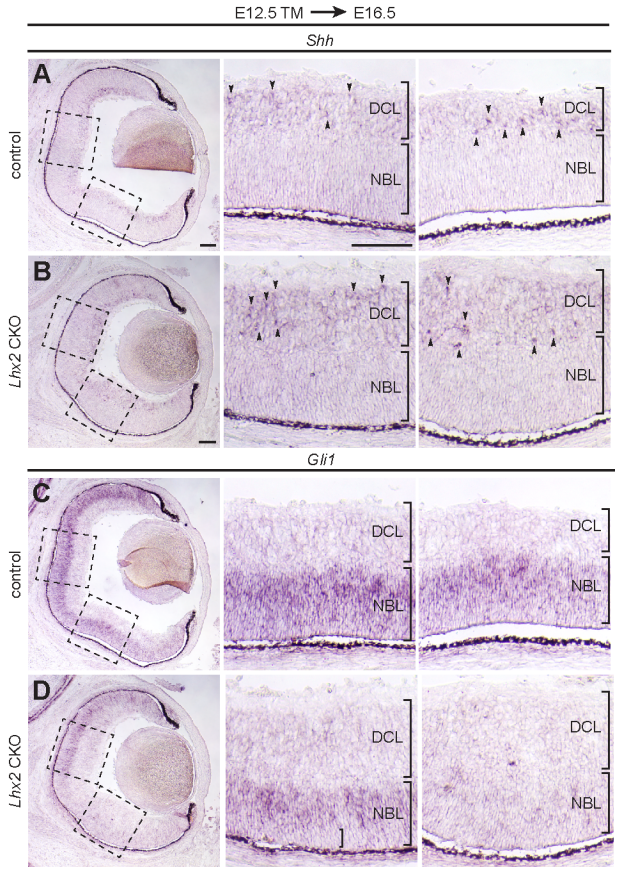

E12.5 TM → E16.5
Shh
A
B
control
Lhx2 CKO
DCL
NBL
Gli1
C
D

Figure 4.3 Ascl1 expression is lost after *Lhx2* CKO, and promoted through Shh pathway activation. (A, B) Two days after tamoxifen treatment, Lhx2 expression is maintained in the vast majority of RPCs within control retinas (A), yet completely lost in *Lhx2* CKO retinas (B). (C, D) In control retinas, Ascl1 is expressed by a subset of RPCs at this age (C), while their number decreases in the ventral retina of *Lhx2* CKO retinas. (E) qPCR confirmation of *Ascl1* loss in E11.5-E15.5 *Lhx2* CKO retinas, which do not display asymmetry in phenotype. (F-G) Vehicle-treated wild-type retinal explants lose expression of *Gli1* after 24 hours in culture (F), but remain still capable of responding to pathway activation via treatment with purmorphamine (Pur) (G). (H-J) The number of Ascl1+ cells increases with Pur treatment. Boxed regions are shown as high magnification panels immediately to the right of all panels. Scale bars: A, F, H, 100 μm; insets, 50 μm.

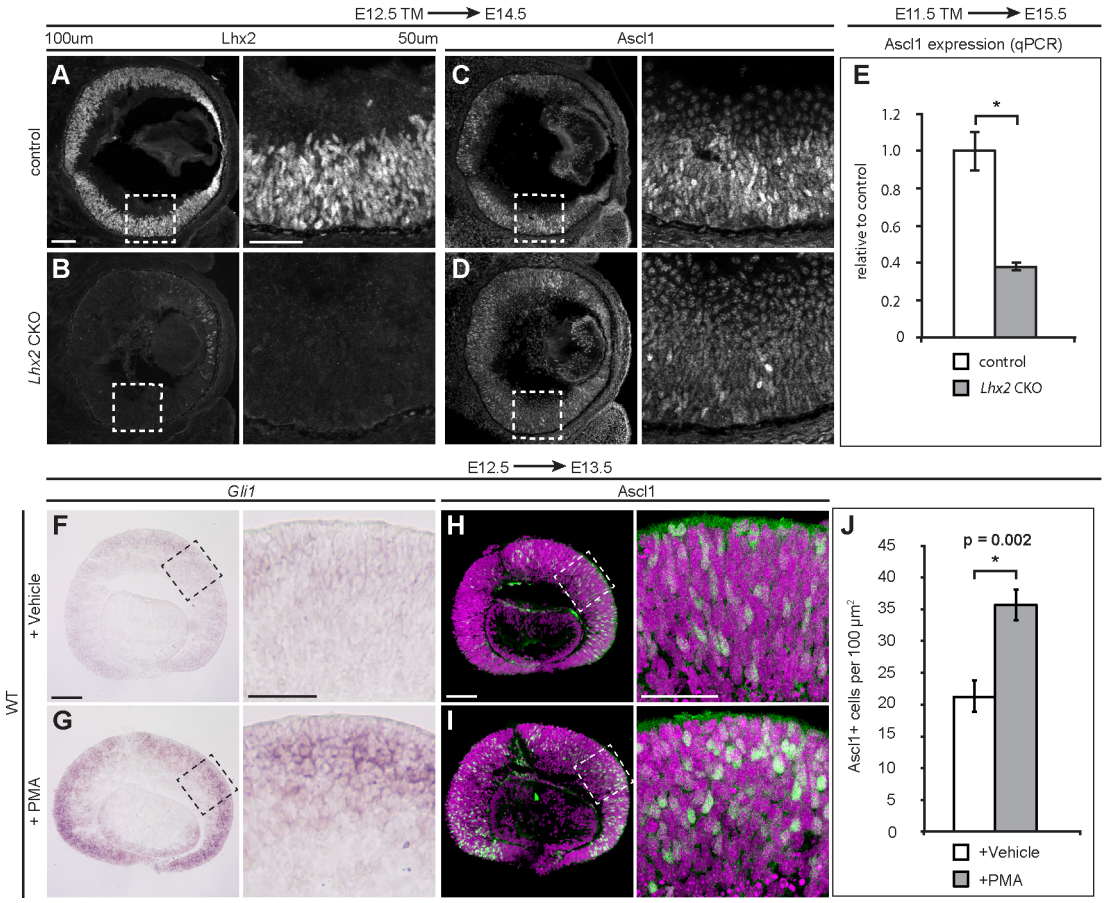
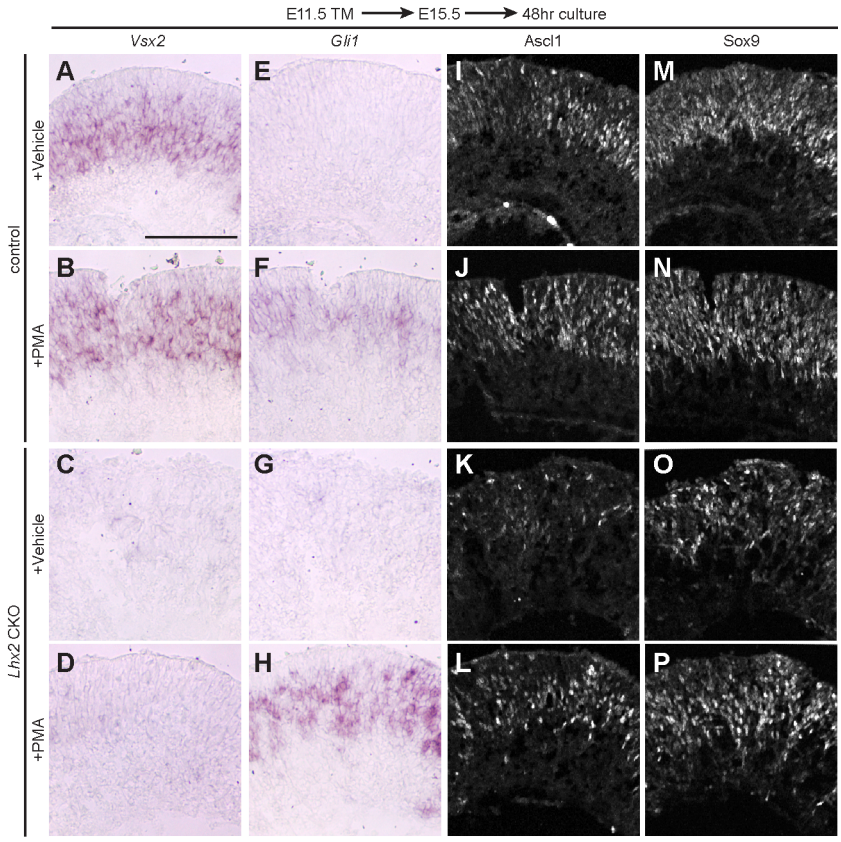

E12.5 TM → E14.5
100um
Lhx2
50um
Ascl1
control
Lhx2 CKO
A
B
C
D
E11.5 TM → E15.5
Ascl1 expression (qPCR)
E
relative to control
1.2
1.0
0.8
0.6
0.4
0.2
0
*
control
Lhx2 CKO
E12.5 → E13.5
Gli1
Ascl1
WT
+ Vehicle
+ PMA
F
G
H
I
J
p = 0.002
*
Ascl1+ cells per 100 µm²
45
40
35
30
25
20
15
10
5
0
+Vehicle
+PMA

Figure 4.4 Shh pathway activation does not rescue RGC overproduction in the *Lhx2* CKO retina. (A-D) While *Vsx2* serves to mark RPCs in control retinas (A, B), its expression is almost completely lost in *Lhx2* CKO retinas (C, D). (E-H) Gli1 expression is lost in retinal explants after a short culture period (E, G) yet restored in both control (F) and *Lhx2* CKO retinas (H) after treatment with Pur. (I-L) EdU birthdating demonstrates RGCs are still produced in control retinas (I) at this stage, and that this rate of production is increased in *Lhx2* CKO retinas (K). Treatment with Pur, however, does not affect the rate of RGC production in either control (J) or *Lhx2* CKO (L) retinas. (M) Quantification of RGC birthdating. Boxed regions are shown as higher magnification insets in the bottom right of relevant panels. Scale bars: A, I, 100 μm; insets, 20 μm.


E11.5 TM
E15.5
48hr culture
Vsx2
Gli1
Ascl1
Sox9
control
+Vehicle
+PMA
Lhx2 CKO
+Vehicle
+PMA
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

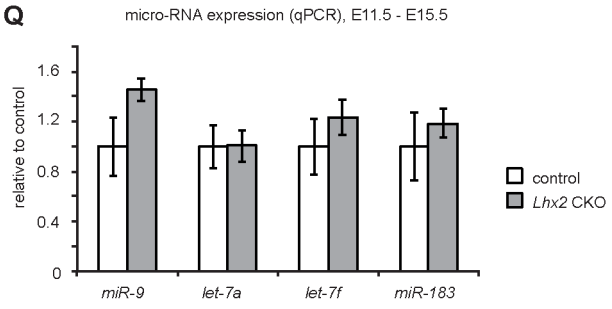

Q
micro-RNA expression (qPCR), E11.5 - E15.5
relative to control
1.6
1.2
0.8
0.4
0
miR-9
let-7a
let-7f
miR-183
control
Lhx2 CKO

Figure 4.5 Shh pathway activation rescues the expression of competence-associated genes in the *Lhx2* CKO retina. (A-D) Vsx2 expression is evident in control retinas (A, B) and almost completely lost in Lhx2 CKO retinas (C, D). (E-H) Gli1 expression is normally lost upon putting retinal explants into culture (E, G), yet restored upon treatment with Pur (F, H). (I, J) In control retinas, Ascl1 expression is evident in a large subset of RPCs at this age (I), and modestly increased with Pur-treatment (J). (K, L) Ascl1 expression is markedly decreased from wild-type levels in *Lhx2* CKO retinas (K), and increased with Pur treatment (L). (M-P) Sox9 expression does not appear to change in control retinas (M, N), yet similar to Ascl1 is decreased in Lhx2 CKO retinas (O) and increased with Pur treatment (P). (Q) qPCR analysis of micro-RNA expression shows no significant changes. Scale bars, 100 μm.

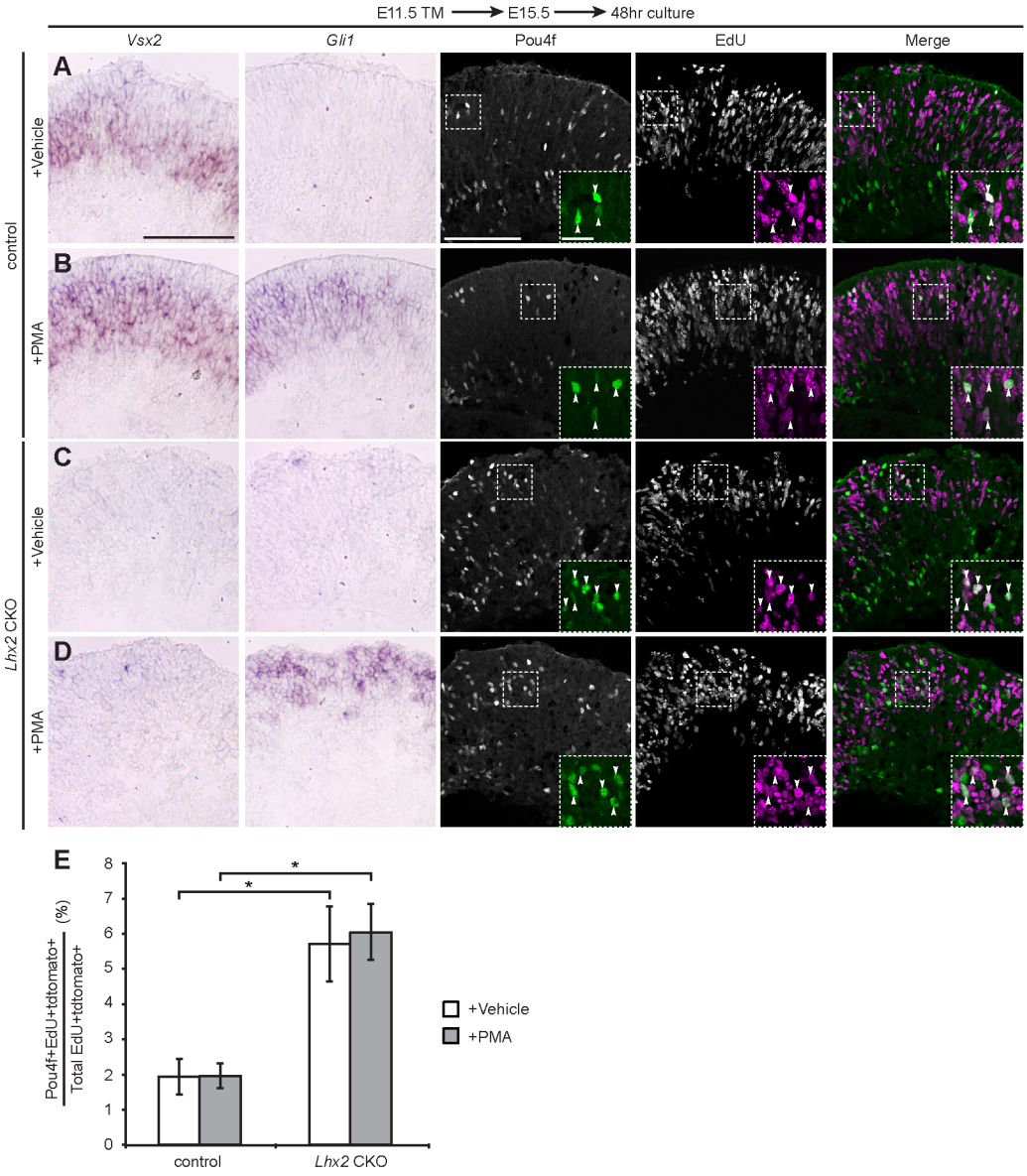

E11.5 TM
E15.5
48hr culture
Vsx2
Gli1
Pou4f
EdU
Merge
A
B
C
D
control
Lhx2 CKO
+Vehicle
+PMA
E
Pou4f+EdU+tdtomato+ / Total EdU+tdtomato+ (%)
control
Lhx2 CKO
+Vehicle
+PMA

Figure 4.6 *Ascl1*^{CreERT2} mediated deletion of *Lhx2* has no effect on proliferation or RGC production. (A, B) When compared to control retinas (A), there are no obvious differences in the number or distribution of tdTomato+ cells in *Lhx2* CKO retinas (B). (A', B') High magnification insets demonstrating that a small percentage of the *Ascl1* lineage contributes to RGC production (arrowheads) in both control (A') and *Lhx2* CKO (B') retinas, as assessed through coexpression of the RGC-specific marker Pou4f. (C) Confirmation that Lhx2 is indeed lost in the majority of tdTomato+ cells. (D-F) Quantifications confirming that there are no significant differences in the total number (D), proliferative fraction (E), or RGC production (F) among tdTomato+ cells of either genotype. Scale bars: A, 100 μm; A', 50 μm.

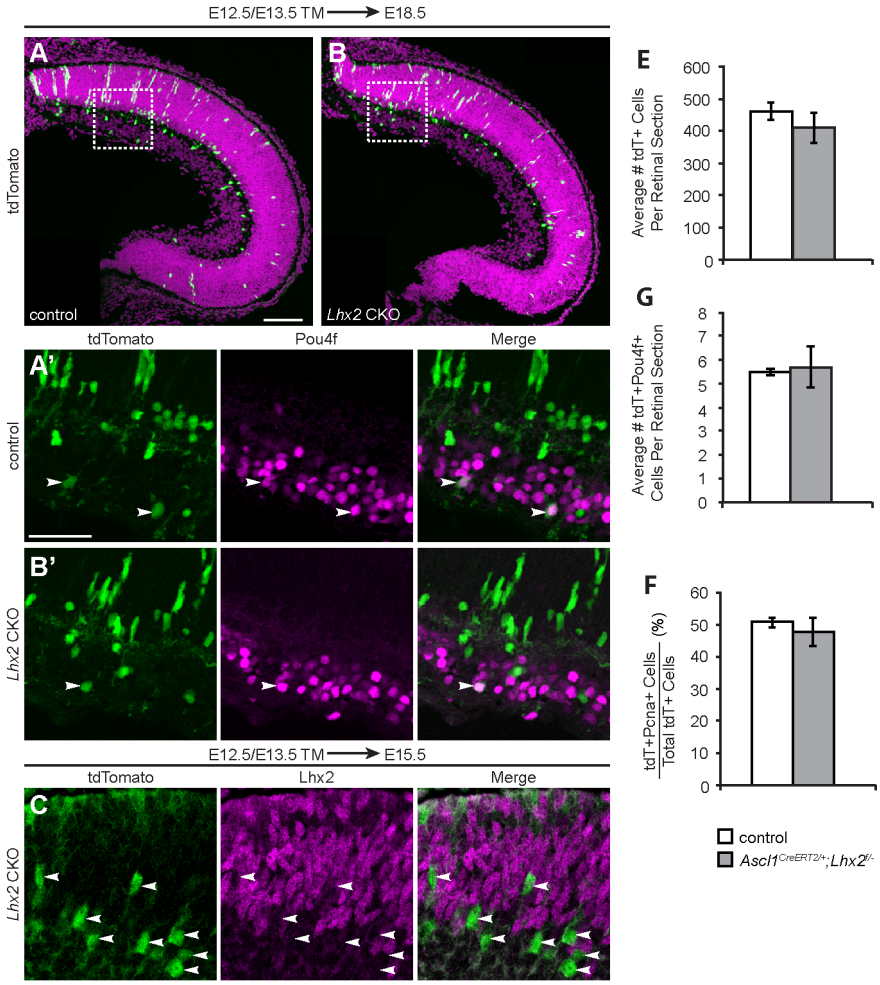
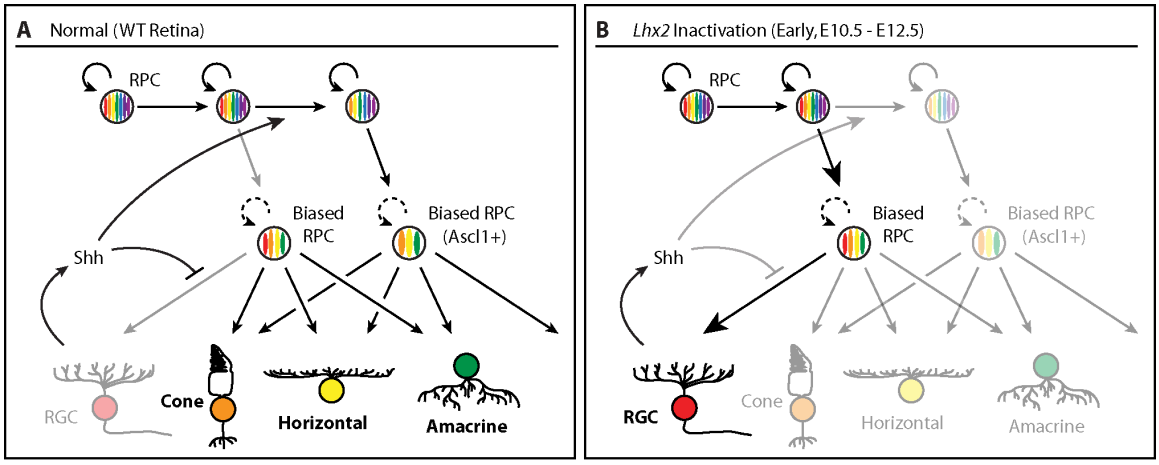

E12.5/E13.5 TM → E18.5
A
B
tdTomato
control
Lhx2 CKO
tdTomato
Pou4f
Merge
A'
control
B'
Lhx2 CKO
E12.5/E13.5 TM → E15.5
tdTomato
Lhx2
Merge
C
Lhx2 CKO
E
Average # tdT+ Cells Per Retinal Section
600
500
400
300
200
100
0
G
Average # tdT+Pou4f+ Cells Per Retinal Section
8
7
6
5
4
3
2
1
0
F
tdT+Pcna+ Cells / Total tdT+ Cells (%)
60
50
40
30
20
10
0
control
Ascl1CreERT2/+;Lhx2f/-

Figure 4.7 Model of *Lhx2* and *Shh* function during early stages of retinal neurogenesis. (A) In the wild-type retina, Lhx2 expression in RPCs allows for a response to RGC-secreted Shh. This further limits RGC production (to the benefit of other early-born cell types) by providing negative feedback through an unknown mechanism. In addition, it promotes a transition in competence through the regulation of Ascl1, presumably leading to the production of later-born cell types. (B) In the *Lhx2* CKO retina, RGC production are *Shh* expression proceed normally, but RPCs are unable to respond to this cue and therefore contribute to a selective and sustained overproduction of RGCs.


A Normal (WT Retina)
RPC
Biased RPC
Biased RPC (Ascl1+)
Shh
RGC
Cone
Horizontal
Amacrine
B Lhx2 Inactivation (Early, E10.5 - E12.5)
RPC
Biased RPC
Biased RPC (Ascl1+)
Shh
RGC
Cone
Horizontal
Amacrine

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CHAPTER 5

DISCUSSION

Summary

The vertebrate retina has long served as a model for the investigation of cell fate decisions in the developing nervous system, and as our knowledge of retinal development grows, it allows us to ask more complex research questions. Accordingly, a tremendous amount was already known, at the outset of these studies, about all of the major retinal cell types and how their individual and coordinated production is regulated during eye development. We sought, however, to address a gap in this knowledge: the function of *Lhx2* in multipotent retinal progenitor cells (RPCs). As with many other genes and signaling pathways, *Lhx2* is used iteratively within a single tissue to accomplish many different and temporally distinct tasks. While many previous studies detailed both the expression and function of *Lhx2* in early eye development (Porter et al., 1997; Zuber et al., 2003; Seth et al., 2006; Viczian et al., 2006; Tétreault et al., 2009; Yun et al., 2009; Hägglund et al., 2011), a role in the regulation of neurogenesis was suspected but unaddressed. Here, we examined this question using conditional inactivation as a loss-of-function approach to selectively remove *Lhx2* expression from RPCs and observe the developmental consequences. We have reported a complex phenotype that indicates several functions.

First, we showed that *Lhx2* is required in RPCs to ensure that an appropriate balance between proliferation and differentiation is maintained throughout neurogenic stages. Inactivation of a conditional *Lhx2* allele (Mangale et al., 2008) using the inducible $Hes1^{CreERT2}$ driver (Kopinke et al., 2011) produced *Lhx2* conditional knock-out (CKO) mice in which premature depletion of the RPC pool led to a corresponding increase in neurogenesis and a selective, stage-dependent production of specific neuronal cell types

(Gordon et al., 2013). This role is in line other studies implicating *Lhx2* in the regulation of progenitor cell behavior (Subramanian et al., 2011; Chou and O'Leary, 2013) and formed a basis for all of our following work.

Second, we demonstrated that this role in regulating proliferation is likely confined to a functional subset of RPCs - those which have committed to differentiation and are close to cell-cycle exit, referred to here as "biased." This was both proposed and examined in conjunction with a reciprocal requirement for the Notch transcription factor *Rbpj* in upstream, stem-like RPCs – referred to here as "unbiased." Conditional inactivation of *Lhx2* and *Rbpj*, both in isolation and combination, yielded gene expression changes consistent with this hypothesis. This led us to formulate a model of RPC diversity which is supported by similar conclusions reached in other recent studies (Brzezinski et al., 2011; Hafler et al., 2012; la Huerta et al., 2012; Suzuki et al., 2013; Cepko, 2014).

Third, and finally, we presented evidence suggesting that the sustained production of retinal ganglion cells (RGCs) observed in *Lhx2* CKO retinas (Gordon et al., 2013) is due to a requirement for *Lhx2* in facilitating the normal response to RGC-secreted SHH, and linked both *Lhx2* and SHH to the promotion of a normal competence transition through regulation of *Ascl1*. These findings are perhaps the most significant, in that they address a longstanding yet unresolved issue in retinal development – the control of competence progression. As with our model of RPC diversity, this led us to a model of *Lhx2* and SHH function that is, again, importantly supported by other recent studies (Brzezinski et al., 2011; Castro et al., 2011; Cwinn et al., 2011; Hufnagel et al., 2013).

Taken together, these results represent a significant advancement in our understanding of both *Lhx2* function and retinal development. In each respective chapter, we have discussed the concerns and implications related to each of the three separate projects. Here, I have placed my results into an increasingly broad context.

Questions and implications

Retina-specific

Early lineage tracing showed that the unbiased labeling of single RPCs invariably gave rise to clones which varied widely in both size and cell type composition (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). While these experiments clearly demonstrated the multipotency of RPCs, they also led to questions regarding equivalence. Specifically, they left open the possibility that distinct populations of RPCs – though unidentified – are allocated to the generation of certain cell types. Currently, at least one line of evidence in the zebrafish retina supports this notion, proposing that distinct lineages are set aside during neurogenesis and allowed to develop in parallel, each contributing to the production of distinct cell types (Vitorino et al., 2009). However, few other studies support such a scenario, and in fact, repeated and systematic analyses of gene expression in the murine retina have failed to identify clearly separable subpopulations of RPC, despite highlighting their heterogeneity (Blackshaw et al., 2004; Livesey et al., 2004; Trimarchi et al., 2008). Rather, several recent studies have converged on a model of RPC diversity that is similar, yet distinct in one important aspect: the timing of lineage restriction. In this model (Fig. 5.1), an upper pool of unbiased RPCs inclined toward proliferation and/or self-renewal continually generates a lower pool of biased RPCs that are limited in proliferative capacity and committed to

differentiation. Immediately prior to cell-cycle exit, many of these RPCs express genes that bias them toward the production of certain cell types (hence the term biased RPC). Thus, while lineage-restricted RPCs do appear to exist, they do not exist as separate cohorts that are set aside and allowed to develop in parallel; instead, the same fate bias seems to arise separately and independently in many different, clonally unrelated RPCs, as they exit the cell-cycle. Further, it seems that combinatorial gene expression is often responsible for this biasing of individual RPCs. This is in line with a collection of studies that demonstrate individual factors are often necessary yet insufficient to ensure the production of certain cell types (Hatakeyama et al., 2001; Prasov and Glaser, 2012; Hufnagel et al., 2013). Logically, this model is more consistent with what is known about retinal development: not only does it explain the variation in clone characteristics cited above, but it allows RPCs to remain flexible in their fate choice until much later – likely a necessity, to allow for coordination in the form of feedback signals, etc. However, there are also aspects of this model that raise certain questions, and must be clarified.

Stochasticity. A quick survey of the field reveals that one of the foremost questions raised by this model is how to reconcile the concept of defined RPC subtypes – each behaving in a predictable manner – with live imaging studies that argue for a stochastic decision-making process (Gomes et al., 2011; He et al., 2012). However, it must be remembered that RPCs were chosen randomly for analysis in these live imaging studies, in contrast with the terminal fate biases detailed above that correlate with gene expression. Perhaps unsurprisingly then, a closer inspection reveals that these reports are not inconsistent with the notion of fate bias in *terminal* divisions. Certain types of terminal division were observed much more frequently than statistically expected,

indicating that bias does exist at that level (Gomes et al., 2011; He et al., 2012). And further, following individual clones over time revealed that sister RPCs were no more likely to generate related lineages than were unrelated, neighboring cells – suggesting a lack of bias above the level of terminal divisions (He et al., 2012). Thus, stochasticity may be incorporated into our model by proposing that it is simply the different subtypes of biased RPC that are generated stochastically, rather than postmitotic cell types.

While this initial reconciliation is satisfying, it simply replaces one set of cell types with another, leaving us without any further understanding of how they are generated in a coordinated manner. In a roundabout way, then, our results force us to address a much more fundamental issue – how apparently stochastic decisions, when utilized in almost countless iteration, can reliably generate a tissue of predictable size and composition. Fortunately, an immediate clarification can begin to simplify this problem: the term "stochastic," as it is used in these reports, does not mean random. Rather than occurring with equal frequency, different outcomes were weighted with relative probabilities in each model (Gomes et al., 2011; He et al., 2012). Instantly, then, it is clear that one of the next major questions in retinal development may be to determine what is setting these probabilities.

At first it seems possible that they may reflect noise or variability in some cell-intrinsic process – for example, a variation in the efficiency of transcription that generates fluctuating levels of an important factor, both above and below a critical threshold. In such a scenario, any given RPC would have a certain probability of expressing that factor above the threshold, and all that do would be guaranteed to make the same decision (e.g., to reenter the cell-cycle). Every single cell, then, would make its decision separately and

independently, with the process resembling a long series of independent coin-flips or die rolls. This has the potential to generate great variation, and as such, any intrinsic basis for these probabilities may fail to address the real issue: how the stochastic behavior of individual cells can be reconciled with the coordination observed across an entire population. Coordination requires more than simply giving each cell the possibility of achieving some outcome - it requires ensuring that only a certain percentage *will* achieve that outcome. Therefore, the relative probabilities used in these stochastic models are more likely to be rooted in a relative rather than an absolute measure – one where cells are compared against each other, and not an absolute metric (e.g., the level of gene expression). In envisioning what such a relative measure might look like, competition for limiting signals becomes an attractive possibility, with the relative probability of making any given decision likely corresponding to an identical probability of receiving or not receiving a certain signal.

One process already proposed to underlie stochastic behavior (Boije et al., 2014), and in line with this model of competition for a signal, is interkinetic nuclear migration (IKNM). During IKNM, the nucleus of every RPC displays an apical-basal migration that occurs in phase with the cell cycle, characterized by mitotic division at the apical membrane, stochastic and basally directed movement during G1 and S phases (due to crowding), and finally, apically directed migration during G2 (Leung et al., 2011). In conjunction with locally restricted or limiting signals (as for the proposed apical-basal gradient in Notch signaling (Del Bene et al., 2008)), this could explain not only individual variability (i.e., stochastic decision-making), but coordination across a population: while any individual cell has a given probability of encountering the signal, it

is simultaneously guaranteed that a certain percentage of the population will encounter the signal. Returning to our model then, the seemingly stochastic generation of differentially biased RPCs may simply occur in response to the need of the tissue, dictated through positive or negative feedback.

Along these lines, future live imaging studies that combine the use of pathway activity reporters and genetically encoded markers of either biased (Almeida et al., 2014) or unbiased (He et al., 2012) RPC subpopulations have the potential to add greatly to our understanding of how RPC diversity is generated. It will also be interesting to discover whether similar mechanisms are used elsewhere within the developing CNS – for example, progenitor cell subtypes in the developing cortex are classified almost exclusively on the basis of their position between, or contact with, the apical and basal membranes (Florio and Huttner, 2014), potentially forming the basis for a similar range of responses to extracellular cues.

Competence. In the model detailed above (Fig. 5.1), biased RPCs are close to cell-cycle exit and directed toward specific fates through the influence of certain genes (e.g., *Olig2*, *Ascl1*, etc.). Thus it is tempting to ask whether these genes are actively conferring competence for those fates. Rather than dictating competence, however, the timing of their expression suggests an *instructive* role; factors that do confer competence are, in contrast, expected to act permissively. Therefore, competence may be defined as the set of these biasing factors (e.g., *Olig2*, *Ascl1*, etc.) that are available to an RPC for expression at any given time. For example, given the lack of its expression before E12.5, Olig2 is likely not available to early-stage RPCs. After E12.5, however, Olig2 is expressed in a subset of RPCs - suggesting that expression is now a possibility, though

not a certainty (Hafler et al., 2012). Which signals or factors actually allow for expression of this or other biasing factors, however, remain unknown. Thus, in addressing the question of what intrinsic factors actually confer competence, our model suggests that at least some of these proneural bHLH genes (e.g., *Olig2*, *Atoh7*, *Ascl1*) - previously considered leading candidates - may need to be removed from consideration. Alternatively, it supports the candidacy of more widely-expressed, upstream factors – such as *Ikaros* (Elliott et al., 2008). However, this discussion highlights another important yet unresolved issue.

Before attempting to identify such factors, it may be pertinent to determine the number and structure of competence states. While competence states are typically portrayed as discrete, and separable (Cepko et al., 1996; Livesey and Cepko, 2001), the reality may be very different (Fig. 5.2). One possibility comes from our model of SHH function, in promoting competence progression through the expression of *Ascl1*. This suggests that competence may represent more of a rolling window, a property that is lost or gained in pieces at either end of a spectrum. Such a scenario is feasible, given the extensive overlap in production observed for most major cell types (Rapaport et al., 2004). Further, instead of conferring competence to generate entire *sets* of cell types, it may be that a collection of different upstream factors simply confer or limit competence to generate *individual* cell types, in a much more piecemeal fashion. Differentiating between such scenarios will of course require further study. However, an examination of the field as it stands now suggests there may be as few as two or three competence states (Fig. 5.2).

In practical terms, the competence of any given RPC is impossible to test or define – no amount of experimentation can determine everything a certain cell *was* capable of. Yet, several different types of experiments allow the observer to infer that an RPC possessed certain levels of competence. In lineage tracing, two-cell clones are often thought to represent terminal divisions, and when composed of two different postmitotic cell types, used to infer that the RPC was competent to generate both fates. In addition, knock-in knock-out experiments using reporter alleles enable the observer to draw conclusions about competence. For example, *Ptf1a* expression is associated with the generation of amacrine and horizontal cells, yet replacement of the coding region with a fluorescent reporter allows the observer to determine that the cells which would have expressed *Ptf1a* are respecified as RGCs (Fujitani et al., 2006). While too numerous to review individually, a steady accumulation of such studies have brought us to a point at which we may at least begin to postulate about the number and structure of competence states. First, it appears that RPCs are competent to produce RGCs, horizontal cells, and amacrine cells. Next, it is likely that they enter a similar state in which they retain horizontal and amacrine cell competence, yet replace the competence to produce RGCs with the competence to produce cones. This is consistent with our results analyzing neurongenic output in the *Lhx2*; *Rbpj* DCKO retina – which suggest that RGC competence and photoreceptor competence may exist separately. Finally, there appears to be a state in which RPCs still retain the competence to generate amacrine cells, yet are also able to generate rods, bipolar cells, and Müller glia. Such a model, proposing only two to three competence states, is fairly accurate, yet not without obvious concerns. Are RPCs not competent to generate both photoreceptor classes at once, given the shared use

of many upstream factors (e.g., *Otx2*, *Crx*, etc.)? Are these competence states inaccurate in their failure to consider the generation of cellular subtypes (e.g., Glycinergic vs. GABAergic amacrine cells, clearly separable by birthdate)? Clearly, such concerns are valid. Yet rather than undermining this model, they serve to highlight its utility, through challenging our understanding and guiding future research.

These discussions help to place our results within the field of retinal development, yet do not encompass all of the current issues. Additional topics currently receiving large amounts of attention include competence progression (Decembrini et al., 2008; 2009; Georgi and Reh, 2010; La Torre et al., 2013), gene regulatory networks (Emerson and Cepko, 2011; Brzezinski et al., 2013; Emerson et al., 2013; Wang et al., 2014), regeneration (Ramachandran et al., 2010; 2012; Pollak et al., 2013), and the derivation of ocular tissues and cell types from pluripotent stem cells (Eiraku et al., 2011; Nakano et al., 2012; Gonzalez-Cordero et al., 2013). A discussion of these, however, is not immediately relevant to our work here. Thus, we forego their consideration to instead briefly consider our results in a different light.

General

While the results presented here do allow us to address specific questions regarding the retina, we must also consider how they contribute to our understanding of development. To do so, we first need to generalize our findings and contrast them with well-established concepts.

Lhx2 as a selector gene. Collective work on *Lhx2* in early eye development (Porter et al., 1997; Zuber et al., 2003; Tétreault et al., 2009; Yun et al., 2009; Roy et al., 2013) converges on a familiar and generalized role for the gene in selecting and

maintaining optic identity during a limited temporal window, similar to its described function as a selector gene in the cortex (Mangale et al., 2008). Selector genes are defined as conferring identity at the level of an organ or tissue at the expense of adjacent fates, and thought to act through defined cis-regulatory elements to simultaneously regulate the expression of many different downstream genes (Mann, 2002). Terminal selector genes are continuously required to maintain these expression patterns, and the properties that result, in differentiated cells (Hobert, 2011). Interestingly, the *Lhx2* ortholog *ttx-3* fulfills this role as a terminal selector for several cell types within the *C. elegans* nervous system (Bertrand and Hobert, 2009; Zhang et al., 2014). In contrast to these studies, we detail here a role for *Lhx2* in helping to define and maintain a transient and multipotent cell type, providing a different view of its function. Through its strict requirement for *Vsx2* expression, *Lhx2* is arguably necessary for conferring RPC identity in a continuous and cell-autonomous manner (similar to a terminal selector gene). However, RPCs are multipotent, and thus instead of requiring the expression of many specific genes, almost require the opposite – the possibility to execute many different programs of gene expression (e.g., those that would define certain postmitotic cell type), yet a continual abstention from commitment. Thus, *Lhx2* most likely functions in a unique capacity to maintain the RPC fate by acting in a permissive, rather than instructive, manner. Several studies have utilized chromatin immunoprecipitation to identify LHX2 binding sites in the genome (Tétreault et al., 2009; Mardaryev et al., 2011; Folgueras et al., 2013; Shetty et al., 2013), and future work in the retina that combines such methods with functional approaches has the potential to reveal exactly how *Lhx2* expression helps to define a multipotent cell type.

Feedback regulation. Feedback plays an essential role in homeostasis and regeneration. However, the role of feedback in development, particularly that of the vertebrate CNS, has received relatively little attention. This is in contrast to the body of work which has attempted to define or establish the *intrinsic* potential of neural progenitor cells (Cayouette et al., 2003; Shen et al., 2006). Nevertheless, modeling work demonstrates clearly how feedback can be used to regulate progression through consecutive steps of a simple and well-defined lineage, whether in situations of homeostasis or development (Lander et al., 2009). Such models are useful. However, they are also simplistic; in reality, cellular lineages are often more complex and less well-defined, with multiple branch points and a capacity for cells to move in multiple directions. As a consequence, it is likely that multiple feedback signals, acting at multiple points in the lineage, are often used to simultaneously coordinate separate aspects of a developmental program (Gokoffski et al., 2011). Indeed, early studies of retinal development proposed that the production of many different cell types may be regulated through negative feedback (Reh and Tully, 1986; Waid and McLoon, 1998; Belliveau and Cepko, 1999). Subsequent work, however, has mainly focused on a single cell type - RGCs (Zhang and Yang, 2001; Kim et al., 2005; Wang et al., 2005; Sakagami et al., 2009). Identifying other feedback signals and clearly defining their context-specific effects on different cell types will be important in neural development moving forward. Our work underscores this point by suggesting that SHH may not only affect RGC production, but competence progression. It is unknown how SHH achieves either of these context-specific functions, yet even after discovering these mechanisms, a major challenge in the field will be to determine how multiple and potentially conflicting

messages (in addition to SHH) are integrated within RPCs. But we are beginning to understand how fate decisions are executed in the retina, and therefore it represents an attractive system for addressing the role of feedback signals in CNS development.

Conclusion

The work presented here, focused around the role of *Lhx2* in RPCs, represents a significant and unique contribution to our knowledge of retinal neurogenesis, speaking to important issues in the field including competence, diversity, lineage-restriction, and feedback. It provides a valuable example of certain ways in which both intrinsic and extrinsic factors can be used to coordinate a population of cells and ensure that a collective goal is reached. Finally, it provides a strong foundation for future work – suggesting unique roles for *Lhx2* and highlighting the importance of feedback regulation in neural development.

Figure 5.1 Model of RPC diversity and fate restriction. (A) An upper pool of unbiased RPCs each possesses the potential to generate all major cell types of the retina (indicated by the various colors), though it is unclear what intrinsic factors limit their competence at any given time point. Their irreversible progression through various competence states, however, results in a corresponding loss of potential. As differentiation proceeds, these unbiased RPCs continually give rise to a changing and diverse pool of biased RPCs. (B) As individual RPCs are specified or selected to differentiate, they are limited to the generation of a subset of cell types (indicated by the various colors), reflecting their competence at the time. In addition, the individual or combined expression of several transcription factors biases them toward the generation of certain cell types within that subset (indicated by relative thickness of the arrows). Specific types of biased RPC depicted are meant to represent examples, many more subtypes of biased RPC are likely specified in parallel.

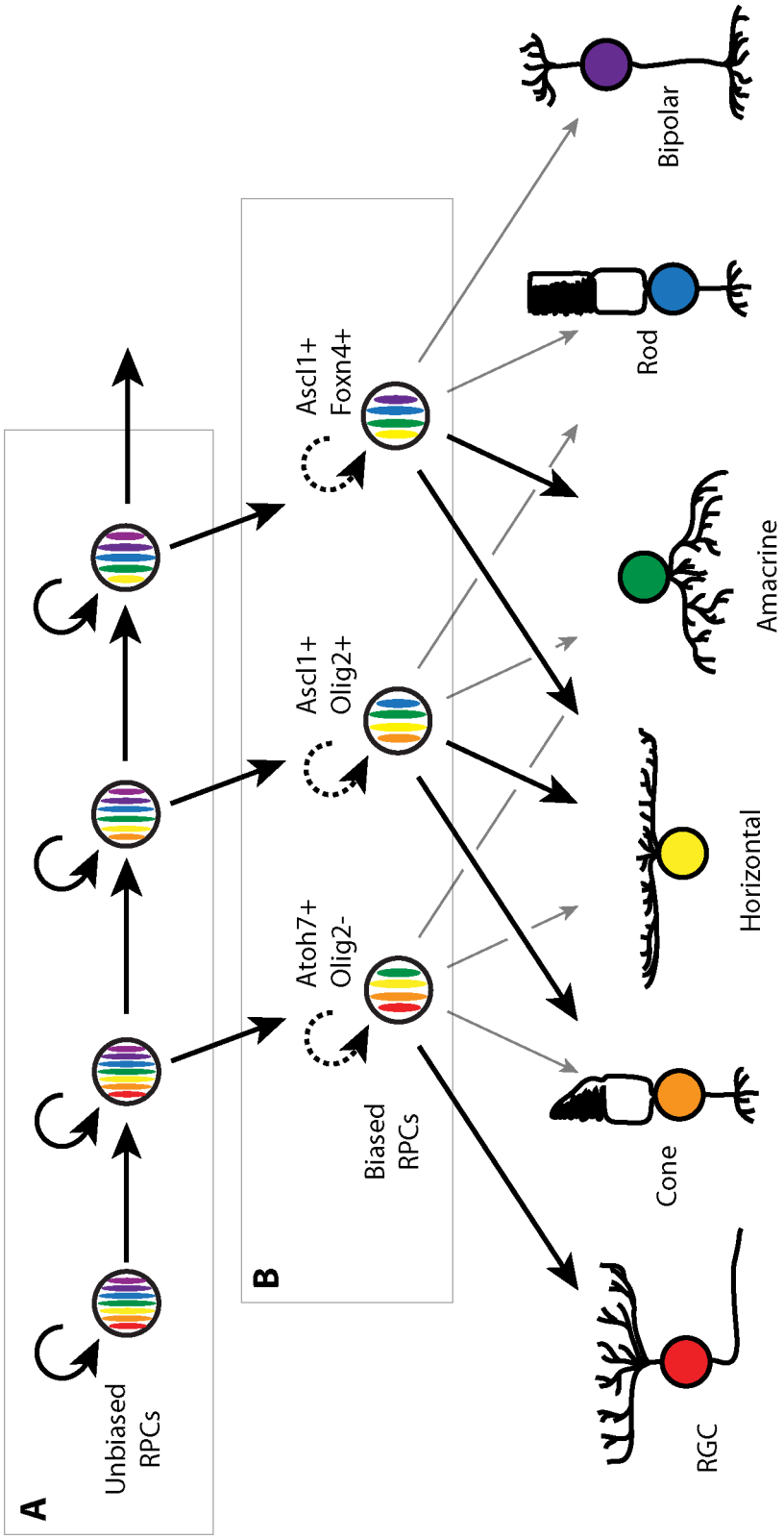
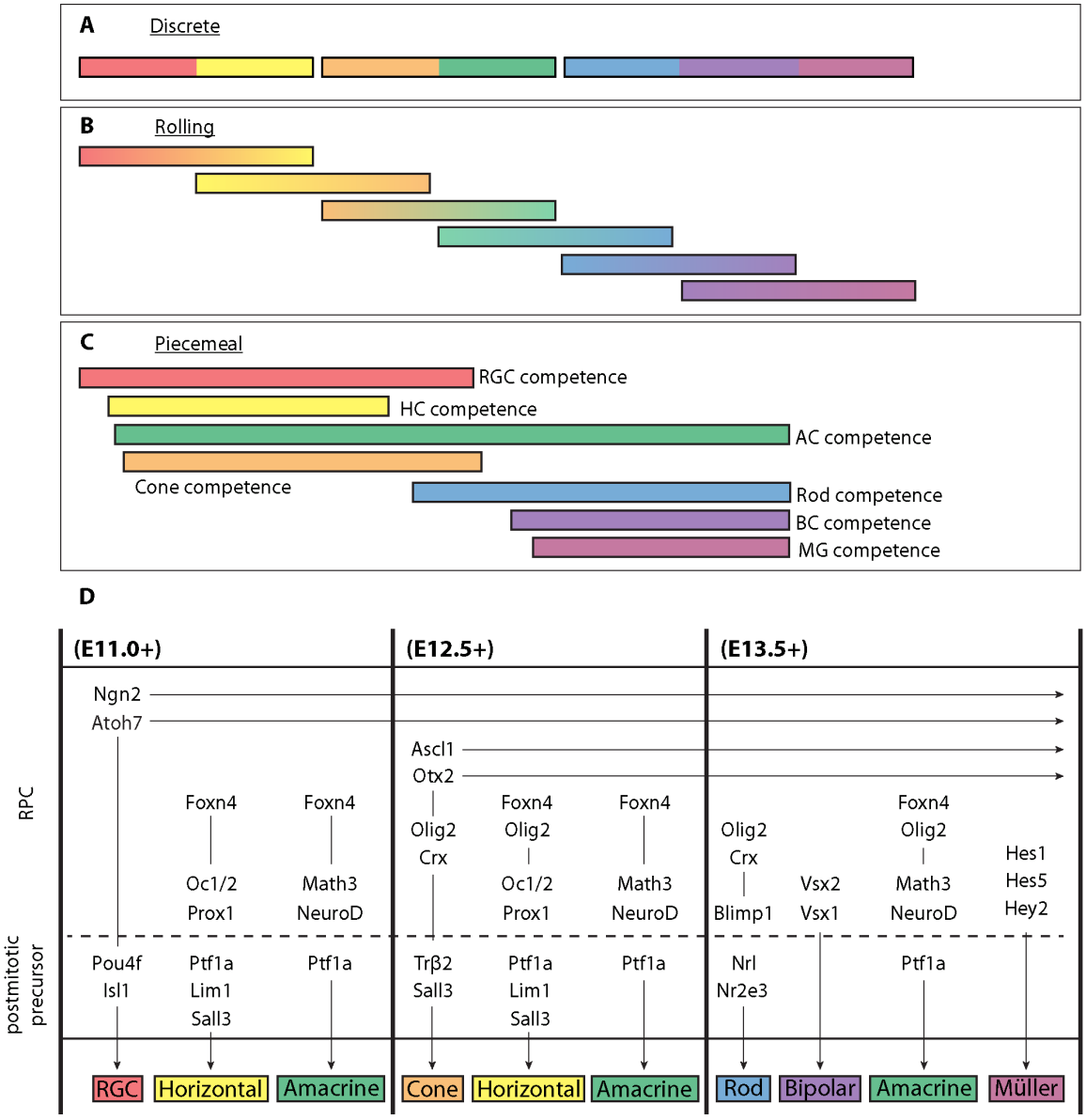

A
Unbiased
RPCs
B
Biased
RPCs
Atoh7+
Olig2-
Ascl1+
Olig2+
Ascl1+
Foxn4+
RGC
Cone
Horizontal
Amacrine
Rod
Bipolar

Figure 5.2 Models of competence state number and structure in the developing retina. (A-C) Several different possibilities exist for structuring the overlap of any given number of competence states. (D) The depiction here is meant to summarize only one of many possibilities for the structure and progression of competence in the vertebrate retina. Vertical arrows are used to associate certain factors with the generation of specific cell types; horizontal arrows, where used, depict continued expression and a contribution to many different cell types as assessed through lineage tracing. Neurogenesis begins with the expression of Ngn2 and Atoh7, with progenitor subsequently occupying a state in which they are competent to generate RGCs, horizontal cells, and amacrine cells. Competence to generate photoreceptors initiates at subsequent stages, requiring the expression of Otx2 and beginning with the production of cones; generation of horizontal and amacrine cells continues. Shortly thereafter, RPCs acquire the competence to produce rods, and in transitioning to postnatal ages, enter a state in which they appear competent to generate the remainder of cell types, including bipolar cells and Müller glia.


A
Discrete
B
Rolling
C
Piecemeal
RGC competence
HC competence
AC competence
Cone competence
Rod competence
BC competence
MG competence
D
(E11.0+)
(E12.5+)
(E13.5+)
Ngn2
Atoh7
Ascl1
Otx2
RPC
postmitotic precursor
Foxn4
Oc1/2
Prox1
Math3
NeuroD
Olig2
Crx
Blimp1
Vsx2
Vsx1
Hes1
Hes5
Hey2
Pou4f
Isl1
Ptf1a
Lim1
Sall3
Trβ2
Nrl
Nr2e3
RGC
Horizontal
Amacrine
Cone
Rod
Bipolar
Müller

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